Carbon dioxide and water transport through plant aquaporins

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Abstract

Aquaporins are channel proteins that function to increase the permeability of biological membranes. In plants, aquaporins are encoded by multigene families that have undergone substantial diversification in land plants. The Plasma membrane Intrinsic Proteins (PIPs) subfamily of aquaporins are of particular interest given their potential to improve plant water relations and photosynthesis. Flowering plants have between 7 and 28 *PIP* genes. Their expression varies with tissue and cell type, through development and in response to a variety of factors, contributing to the dynamic and tissue specific control of permeability. There are a growing number of PIPs shown to act as water channels, but those altering membrane permeability to CO_2 are more limited. The structural basis for selective substrate specificities has not yet been resolved, although a few key amino acid positions have been identified. Several regions important for dimerization, gating and trafficking are also known. PIP aquaporins assemble as tetramers and their properties depend on the monomeric composition. PIPs control water flux into and out of veins and stomatal guard cells and also increase membrane permeability to CO_2 in mesophyll and stomatal guard cells. The latter increases the effectiveness of Rubisco and can potentially influence transpiration efficiency.

Summary statement

Aquaporins are intrinsic membrane proteins encoded by a multigene family that function to increase membrane permeability to water, CO_2 and other molecules. There are many levels of regulation (e.g. diurnal expression, membrane targeting, tetramer composition, gating) that result in highly dynamic and tissue specific control of permeability. Owing to the ease of measurement, aquaporins associated with water permeability have been more extensively characterised than those facilitating CO_2 transport. Manipulation of aquaporins is actively being explored in efforts to improve plant performance with respect to plant water relations, stomatal function and photosynthesis.

Introduction

During photosynthesis, CO_2 assimilation reduces the partial pressure of CO_2 inside chloroplasts relative to that in the surrounding atmosphere. As a result, there is a net diffusion of CO_2 into the leaf. At the same time, water diffuses from wet mesophyll cell wall surfaces out to the drier atmosphere. Plants control this diffusive exchange of water and CO_2 by enclosing leaves with an impermeable barrier perforated by dynamic pores called stomata. CO_2 uptake from the atmosphere is inevitably linked to transpirational water loss because water and CO_2 share the same diffusional path across the epidermis through stomata. A wall surrounds each plant cell that defines its shape and allows hydraulic pressure to build up within it. The porous matrix of the wall contains an aqueous solution that allows water and gases to pass between the inside of the cell and the atmosphere. Behind each cell wall lies the plasma membrane. This lipid bilayer contains almost equal proportions of phospholipids and sterols while proteins account for about 40% of the mass (Yoshida & Uemura, 1986). The plasma membrane provides cells with a barrier that limits the exchange of water, solutes and gases between the external and internal solution. Exchange across the membrane is then controlled by protein pores and transporters that are integrated into it.

In mammalian red blood cells, there is an abundant 28kD integral membrane protein (CHIP28, now called AQP1) which was first cloned in 1991 (Preston & Agre, 1991). The function of this protein as a water channel was discovered by expressing it in *Xenopus* oocytes (Preston *et al.*, 1992). Oocyte membrane water permeability containing CHIP28 could be inhibited by $HgCl_2$. Using the same expression system, two other proteins homologous to CHIP28 were found to be water channels, one from the collecting duct of the rat kidney (Fushimi *et al.*, 1993) and the other a tonoplast intrinsic protein (TIP) from *Arabidopsis thaliana* (Maurel *et al.*, 1993). Given that these proteins came from a large family of integral membrane proteins identified in diverse organisms and the demonstration that all three acted as water channels, the term aquaporin was coined (Agre *et al.*, 1993). Since then, there has been an explosion of research on aquaporins and numerous reviews on their role in plants (Chaumont & Tyerman, 2014, Heinen *et al.*, 2009, Javot & Maurel, 2002, Kaldenhoff & Fischer, 2006, Katsuhara *et al.*, 2008, Maurel *et al.*, 2015, Maurel *et al.*, 2008, Moshelion *et al.*, 2015, Prado & Maurel, 2013, Tyerman *et al.*, 2002).

This review focuses on aquaporins in the plasma membrane that alter permeability to water and CO_2. We first introduce the phylogenetic origins of major intrinsic proteins before considering the evidence that they are water channels. The role of aquaporins varies depending on the location in the plant.

Evidence for aquaporins altering the membrane permeability to CO_2 is more limited than for water, but there is evidence that CO_2 permeable aquaporins function in leaves both in mesophyll cells and stomatal guard cells. We consider the functionally significant amino acids and end with a brief summary of attempts to engineer improved plants by manipulating aquaporins.

Phylogenetic background to Major Intrinsic Proteins

Major Intrinsic Proteins (MIPs) are an ancient superfamily of proteins found essentially throughout all taxonomic kingdoms. Their classic six transmembrane structure consisting of two repeated halves evolved through a tandem intragenic duplication of a three transmembrane domain protein that may have functioned as a homodimer (Park & Saier, 1996). The first major phylogenetic division of MIPs is considered to be the separation into water channel aquaporins (AQPs) and glycerol transporting aquaglyceroporins (GLPs) within bacteria and archaea. An increased availability of genomic sequences has advanced this view showing a more complex diversification in bacteria and archaea involving four major grades; GLP, AQPM, AQPZ and the newly identified AQPN (Finn *et al.*, 2014). Since then, these groups have undergone an explosion in sequence and functional diversification resulting in many types (subfamilies) and variants (groups and isoforms therein) of MIPs across all kingdoms (Abascal *et al.*, 2014, Finn & Cerda, 2015). Aquaporins are most diverse in green plants (Viridiplantae), with twelve distinct subfamilies acknowledged thus far (Figure 1); MIPsA-E, GIP (GlpF-like intrinsic proteins), HIP (hybrid intrinsic proteins), PIP (plasma membrane intrinsic proteins), TIP (tonoplast intrinsic proteins), NIP (NOD26-like intrinsic proteins), SIP (small basic intrinsic proteins), XIP (uncharacterised X intrinsic proteins).

Nomenclature of the subfamilies is somewhat arbitrary being based on a combination of main cellular localisation (PIPs, TIPs), first identified tissue expression (NIPs,(Heymann & Engel, 1999)), protein structure (SIPs, (Johanson & Gustavsson, 2002); HIPs, (Danielson & Johanson, 2008)), phylogenetic relationships with earlier identified aquaporins (GIPs, (Gustavsson *et al.*, 2005); MIPsA-E, (Anderberg *et al.*, 2011)) or simply uncharacterised (XIPs – (Danielson & Johanson, 2008)). Little is known about the localisation and functions of MIPsA-E or HIPs, but the other subfamilies have been well characterised. GIPs localise to the plasma membrane and transport glycerol but not water (Gustavsson *et al.*, 2005). PIPs generally localise to the plasma membrane but have been found to co-localise to the chloroplast envelope (Beebo *et al.*, 2013, Uehlein *et al.*, 2008) and are highly water permeable, but can also transport hydrogen peroxide and uniquely carbon dioxide (Bienert *et al.*, 2007, Hooijmaijers *et al.*, 2012, Kaldenhoff *et al.*, 2014, Prado & Maurel, 2013, Tian *et al.*, 2016). TIPs are mainly localised in the tonoplast (vacuole membrane), but have been found in the plasma, chloroplast and thylakoid membranes (Beebo *et al.*, 2013, Ferro *et al.*, 2010) and are permeable to water and other solutes such as urea, ammonia and hydrogen peroxide (Dynowski *et al.*, 2008, Liu *et al.*, 2003, Loque *et al.*, 2005, Mao & Sun, 2015). The co-localisation of PIPs and TIPs to the chloroplast and thylakoid membranes maybe particularly import in supplying water and CO_2 for photosynthetic reactions (discussed later). XIPs are plasma membrane localised and permeable to various solutes but only moderately to water (Bienert *et al.*, 2011, Lopez *et al.*, 2012). SIPs are localised to the endoplasmic reticulum (ER) and have moderate water permeability (Ishikawa *et al.*, 2005, Noronha *et al.*, 2014). NIPs are found in the plasma membrane and ER and are permeable to numerous substrates including both beneficial and toxic metalloids, but generally show poor water permeability (Pommerrenig *et al.*, 2015).

The most basal of green plants, the green algae (chlorophytes), have seven aquaporin subfamilies; MIPsA-E, which are specific to algae, in addition to PIP, GIP and SIP homologs (Abascal *et al.*, 2014, Anderberg *et al.*, 2011). These aquaporins are found in sparse and assorted complements among species which may reflect redundant functions or specific lifestyle requirements given the remarkable variation in green algal ecology (Anderberg *et al.*, 2011, Leliaert *et al.*, 2012). In land plants (Embryophyta), the number of aquaporin subfamilies has contracted from seven in primitive land plants to five in seed plants (Spermatophyta). However, the number of groups and isoforms therein has undergone substantial expansion and diversification (Figure 1). Gone from higher plants are the HIPs and GIPs, presumably due to functional redundancy with the TIPs and NIPs, respectively (Abascal *et al.*, 2014, Anderberg *et al.*, 2012). XIPs persist in higher plants to an extent, having been lost in the Brassicaceae, monocots and possibly the gymnosperms, but diversified in the Malpighiales. The SIPs split into two groups with the emergence of angiosperms, but in general show little diversification compared to the other subfamilies. The TIPs, may have ancestral ties with the algal MIPA, but emerged as a distinct subfamily in land plants (Anderberg *et al.*, 2011). An independent diversification in primitive plants gave rise to the TIP6 group, while the major expansion seemingly originates in the ancestor of seed plants after divergence from the ferns. TIP2, TIP3 and TIP4 represent the basal groups, with TIP1 and TIP5 emerging in angiosperms as sister groups to TIP3 and TIP2 respectively (Abascal *et al.*, 2014, Laur & Hacke, 2014). Coinciding with their wide substrate specificities, NIP isoforms are the most divergent among higher plant aquaporin subfamilies. This makes it difficult to fully resolve the relationships between groups of distantly related species using subgrouping normally applied to other aquaporin subfamilies (Pommerrenig *et al.*, 2015). Using the alternative ar/R filter classification shows the ancestral NIPs belong to groups II and III, with the latter having proliferated in monocots and been lost in the brassica genus. Group I NIPs are seed plant specific. The PIP subfamily has the longest clearly discernible lineage among plant aquaporins. PIP isoforms exist in the chlorophytes and have key similarities to algal MIPEs suggesting a paralogous and possibly redundant relationship; consistent with PIPs and MIPEs not being found in the same extant algal species (Anderberg *et al.*, 2011). PIPs diverge into two highly conserved groups (PIP1 and PIP2) prior to the emergence of land plants and although there has been no further expansion of group types, there has been a substantial proliferation in the number of PIP1 and PIP2 isoforms coinciding with the appearance of seed plants.

Horizontal gene transfer (HGT) has been important in the acquisition of aquaporins into the plant kingdom. The origin of GIPs reside in a HGT event with bacteria Glp (Gustavsson *et al.*, 2005), while the ancestral NIP arose through a HGT event involving bacterial AQPN (Finn & Cerda, 2015). HGT events would also, in part, account for the assorted complements of MIP types found in different chlorophytes (Figure 1; (Anderberg *et al.*, 2011)). In land plants, the impressive diversification of aquaporins has likely been facilitated by a propensity for gene duplication events, especially prevalent in the angiosperms. Major diversification of land plant aquaporins coincide with ancient polyploidisation events occurring prior to the divergence of spermatophytes and another before angiosperms arose (Figure 1; (Jiao *et al.*, 2011)). This association with events that heralded today's diverse plant life, supports aquaporins being important in higher plant evolution. Later lineage-specific polyploidisation events likely facilitated further diversification and expansion apparent between angiosperm lineages (Abascal *et al.*, 2014). Reflecting this concept, aquaporin numbers are often higher in species that have experienced a more recent polyploidy event (e.g. Soybean vs Common bean; *Arabidopsis* vs *Brassica rapa*; Poplar vs Castor bean; Figure 1). In addition to whole genome duplications, single gene replication events locally (i.e. tandem or proximal duplication) or between different regions of the genome (transposition or retrotransposition) have contributed to lineage and species specific expansion of aquaporins.

As with most genes, the aquaporin duplicates (homeologs or paralogs) would be deleted over time. Progress of such gene loss is evident in the numerous aquaporin pseudogenes present in soybean, cotton and cabbage which have undergone relatively recent polyploidisation (Diehn *et al.*, 2015, Park *et al.*, 2010, Zhang *et al.*, 2013). Retention of a paralog relies on the duplicate gene acquiring a novel role (neo-functionalisation) or the two duplicate genes dividing the original function (sub-functionalisation) (Rensing, 2014). Given the high homology of aquaporin isoforms in a given species, it is likely that paralog retention has largely been driven through divergence in gene regulation. Consistent with this, aquaporin isoforms within a species show differences in expression patterns even at the broad level of whole tissues (e.g. leaf, roots, fruit, seed etc.) (Abascal *et al.*, 2014, Ariani & Gepts, 2015, Cohen *et al.*, 2013, Diehn *et al.*, 2015, Gupta & Sankararamakrishnan, 2009, Hu *et al.*, 2015, Martins *et al.*, 2015, Quigley *et al.*, 2001, Reddy *et al.*, 2015, Reuscher *et al.*, 2013, Sakurai *et al.*, 2005, Tao *et al.*, 2014, Venkatesh *et al.*, 2013, Zou *et al.*, 2015b, Zou *et al.*, 2016); with differences becoming more distinct at the level of cell types within a tissue (Alexandersson *et al.*, 2010, Bots *et al.*, 2005, Fraysse *et al.*, 2005, Gomes *et al.*, 2009, Hachez *et al.*, 2006, Lopez *et al.*, 2012, Prado *et al.*, 2013, Sakurai *et al.*, 2008). Divergence in aquaporin isoform expression also extends to differential responses to abiotic stresses and environmental stimuli (see later section on regulation of aquaporin expression). Presumably this refined transcriptional regulation offers plants an intricate control over hydraulic properties and transport of the various solutes and gases that permeate aquaporins.

With roles in water transport and nutrient acquisition, it is not surprising that the evolution of aquaporins and plants appear strongly intertwined. Aquaporin diversity in bryophytes compared to algal species may represent an additional need for aquaporins to transition from an aquatic to a terrestrial environment (Anderberg *et al.*, 2011, Danielson & Johanson, 2008, Hanson & Rice, 2014). Subsequent aquaporin diversification in land plants likely stems from an increasing complexity of organ structures and cell types, involved in migration from low growth habits in moist environments to taller growth and more arid conditions. The association of TIPs with the metabolically important vacuole and ability of NIPs to transport nutritionally beneficial metalloids (e.g. boron and silicon) (Martinoia *et al.*, 2007, Pommerrenig *et al.*, 2015) likely drove their diversification during land plant evolution. Early evolution of PIPs in plants reflects the primary importance of water transport. PIPs in free living aquatic algae may regulate water content for buoyancy, aiding in nutrient acquisition and transport (Anderberg *et al.*, 2011, Komsic-Buchmann *et al.*, 2014, Raven & Doblin, 2014), while in the earliest land plants, PIP proteins provide osmoprotection (Lienard *et al.*, 2008). In higher plants, water permeability has continued as a key selected trait evident by strong PIP gene expression in tissues with large water fluxes (e.g. vasculature, stomata, flowers). Uniquely among plant aquaporins, PIPs also transport CO_2, the substrate for carbohydrate production via photosynthesis. In animals, AQP 0, 1, 4, 5 and 6 and bacterial AQPZ in cyanobacteria PCC7942 have also been shown capable of transporting CO_2 to varying levels (Ding *et al.*, 2013, Geyer *et al.*, 2013, Musa-Aziz *et al.*, 2009). These animal aquaporins, AQPZ and plant PIPs appear to have a deep rooted phylogenetic connection (Abascal *et al.*, 2014), that temptingly points to an ancient origin for CO_2 specificity. However, this would require assuming many independent functional losses of CO_2 specificity given its infrequency compared to water permeability. It is more likely that CO_2 specificity arose through convergent evolution, with the water permeable aquaporin architype reasonably amenable to sequence variation leading to significant CO_2 permeability that is selected upon if the need arises.

In aquatic environments, CO_2 diffusion is much slower than in terrestrial environments and bicarbonate (HCO_3^-) is frequently the dominant inorganic carbon species (Wang *et al.*, 2015). Consequently algae have evolved both active CO_2 and HCO_3^- uptake systems (Wang *et al.*, 2015), making CO_2 permeable MIP/PIP unnecessary or even undesirable. A niche role may still exist as *Chlamydomonas* possess a plasma membrane localised passive CO_2 channel in the form of Rhesus proteins that reportedly aid carbon uptake under elevated CO_2 conditions (Soupene *et al.*, 2004, Soupene *et al.*, 2002). However, the absence of Rhesus proteins in land plants and their existence in algae (Peng & Huang, 2006), may yet represent a further preclusion for an algal CO_2 MIP/PIP. A role for a CO_2 permeable PIP would presumably be most favoured in a terrestrial environment. Mesophyll CO_2 conductance in land plants generally scales with their phylogenetic ranking (Flexas *et al.*, 2012, Raven & Beardall, 2016, Tosens *et al.*, 2016). Low values for bryophytes through to ferns is predominantly linked with sub-optimal anatomical traits and requirements for external moisture coatings (Field *et al.*, 2015, Flexas *et al.*, 2012, Kubásek *et al.*, 2014, Raven & Beardall, 2016, Royles *et al.*, 2013, Tosens *et al.*, 2016). For a given cell wall thickness, ferns have a mesophyll conductance per unit of chloroplast surface area exposed to intercellular airspace that is similar to angiosperms. This suggests that the extent of PIP facilitated CO_2 diffusion in ferns is similar to that in angiosperms despite having an apparently more limited number of PIP genes. The diversification of PIPs in seed plants enabled temporal and spatial specialisation of expression, coinciding with greater photosynthetic energy demands, more complex leaf structures and decreasing atmospheric CO_2 concentrations (Beerling, 2005). A better understanding of PIP (or aquaporins in general) evolution could be achieved by complementing protein sequence phylogenetic reconstructions with substrate specificities from more aquaporins across the plant kingdom.

Evidence that plant aquaporins enhance membrane permeability to water

The demonstration that a plant TIP acted as a water channel (Maurel *et al.*, 1993) spurred a search for other candidate genes encoding aquaporins in the plasma membrane. Using a mammalian COS cell line and an antibody raised against plasma membrane proteins, 5 genes from *Arabidopsis* representing 2 families of PIPs were identified (Kammerloher *et al.*, 1994). These were expressed in *Xenopus* oocytes to demonstrate that they were water channels. The PIP2 type showed greater water permeability than the

PIP1 type, but both types could be inhibited by $HgCl_2$. Since plasma membrane composition is likely to vary between organisms, the function of an aquaporin in one cell type does not necessarily mean that it will function in the same way in another cell. Thus it is important to demonstrate that aquaporins alter water permeability *in planta*. To do this, an antisense line was created against PIP1b in *Arabidopsis* (now AtPIP1;2) driven by the cauliflower mosaic virus 35S promoter (Kaldenhoff *et al.*, 1998). Protoplasts prepared from leaf mesophyll tissue were found to have greatly reduced permeability to water compared to protoplasts isolated from control plants. Antisense-pip1b plants also developed a five-fold greater root mass than control plants that presumably compensated for their impaired water uptake capability.

An antisense construct against NtAQP1 with a cauliflower mosaic virus 35S promoter was introduced into *Nicotiana tabacum* (Siefritz *et al.*, 2002). Protoplasts isolated from the roots of these plants showed reduced permeability to water. Hydraulic conductivity of intact roots was also reduced by 55%. In contrast to *Arabidopsis* antisense-AtPIP1b plants, no change in the relative size of the root system was observed for tobacco, but the antisense plants were more prone to wilting compared to wild type.

Membrane permeability depends on temperature and the response can be described by the Arrhenius activation energy, E_a. When water is diffusing through the lipid bilayer, a high E_a is expected and when flow occurs through a channel, a low E_a is expected. When human AQP1 was expressed in *Xenopus* oocytes, E_a decreased from >> 10 kcal mol^{-1} for control oocytes to < 3 kcal mol^{-1} for oocytes injected with AQP1 (Preston *et al.*, 1992). Similarly, the E_a for tonoplast enriched vesicles isolated from suspension cultured *Nicotiana tabacum* containing active aquaporins (i.e. sensitive to $HgCl_2$ inhibition) was 2.5 kcal mol^{-1} compared to 13.5 kcal mol^{-1} for plasma membrane enriched vesicles (Maurel *et al.*, 1997). The water permeability of purified plasma membrane vesicles isolated from *Arabidopsis* grown in suspension culture could be substantially reduced by the presence of Ca^{2+} (Gerbeau *et al.*, 2002). At the same time, the permeability was rendered much more responsive to temperature, consistent with lipid mediated water diffusion brought about by the closure of a channel. In the absence of Ca^{2+}, the permeability was sensitive to pH, declining to minimal values below pH 7. These findings demonstrated that plant aquaporins could be gated and their permeability could be altered by changes in Ca^{2+} and pH (see later section on gating).

PIP aquaporins as water channels

The relative ease of expressing specific aquaporins in *Xenopus* oocytes to assess their function has led to a growing list of genes from a range of plant species that are capable of increasing membrane water permeability when expressed in *Xenopus* oocytes. For some species, multiple isoforms have been shown to function as water channels (Table 1). In the case of *Arabidopsis,* the impact on water permeability was greater for PIP2s compared to PIP1s e.g. (Kammerloher *et al.*, 1994). Weaker effects were also observed for PIP1s compared to PIP2s from *Hordeum vulgare* (Katsuhara & Shibasaka, 2007), *Oryza sativa* (Li *et al.*, 2000, Lian *et al.*, 2004) and *Raphanus sativa* (Suga & Maeshima, 2004). However, the observation that PIP1s were apparently less effective than PIP2s came into question following the discovery that co-expression of PIP1 with PIP2 genes in *Xenopus* oocytes increased water permeability (Fetter *et al.*, 2004). Oocytes with co-expression of PIP1 and PIP2 genes had greater water permeability than oocytes injected with the same amount of PIP2 genes but no PIP1 genes, and PIP1 genes by themselves had no effect. Previously it had been established that PIP1 proteins could be recovered from the plasma membrane fraction of *Xenopus* oocytes when just PIP1 genes were injected, but these did not alter membrane permeability to water (Chaumont *et al.*, 2000). Thus, to become functional in the plasma membrane of *Xenopus* oocytes, PIP1 needed to assemble in a hetero-tetrameric association with PIP2 (Fetter *et al.*, 2004). Subsequently, when PIP1 genes have been co-expressed with PIP2 genes, increased water permeability has been associated with PIP1s from a range of species (Table 2). For both *Zea mays* (Fetter *et al.*, 2004) and *Hordeum vulgare* (Horie *et al.*, 2011), multiple forms of PIP2 were capable of conferring functionality to the PIP1 protein. ZmPIP1;2 was also capable of gaining functionality through association with a PIP2 from another species: AtPIP2;3 (Fetter *et al.*, 2004). Perhaps even more surprising was the observation that co-expression of ZmPIP1;1 with ZmPIP1;2 also resulted in increased permeability of *Xenopus* oocytes to water (Fetter *et al.*, 2004). In this case, functionality was presumably enabled through the formation of a hetero-tetramer between ZmPIP1;1 and ZmPIP1;2. Not all forms of PIP1 increase water permeability when co-expressed with PIP2. For HvPIP1;3, co-expression with any one of HvPIP2;1-5 could not influence permeability whereas water permeability could be further increased when HvPIP2;1-5 was co-expressed with either HvPIP1;2 or HvPIP1;4 (Horie *et al.*, 2011).

Different aquaporin monomers can dimerise through a disulphide linkage in loop A before forming into tetramers (Bienert *et al.*, 2012). The functional and regulatory properties of heterotetramers reflect their composition and may provide an additional level of control (Yaneff *et al.*, 2014). Expression profiling has revealed that certain combinations of PIP1 and PIP2 genes occur in *Arabidopsis,* maize and rice (Yaneff *et al.*, 2015). An F220A mutation in the transmembrane domain 5 of ZmPIP1;2 activated its water activity while inactivating its dimeric partner ZmPIP2;5 within a heterotetramer (Berny *et al.*, 2016).

Finally, it is worth repeating that water permeability associated with a given PIP gene depends on the membrane system it is tested in. For some species, PIP1 can form tetramers and reach the plasma membrane in yeast (Sabir *et al.*, 2014) or *Xenopus* oocytes (Berny *et al.*, 2016, Chaumont *et al.*, 2000) without requiring an association with PIP2. AQP1 from tobacco was shown to enhance water permeability in *Xenopus* oocytes (Biela *et al.*, 1999) but not when expressed in yeast (Otto *et al.*, 2010). For HvPIP1;2 expressed in *Xenopus* oocytes, water permeability was only observed when it was co-expressed with PIP2 (Horie *et al.*, 2011) whereas it conferred increased water permeability by itself when expressed in yeast (Besse *et al.*, 2011). A PIP1 from *Lilium longiflorum* increased water permeability weakly when expressed in *Xenopus* oocytes, whereas when expressed in *Nicotiana tabacum* under a cauliflower mosaic virus 35S promoter, it greatly increased mesophyll protoplast permeability to water (Ding *et al.*, 2004). The large number of aquaporin genes and the tissue specificity of their expression make it a challenge to conclusively attribute function *in planta*.

Path of water movement through the plant

The majority of water used by plants is extracted from the soil by roots and transported to the shoot where it is lost to the atmosphere by transpiration from leaves. Long distance water transport occurs through dead xylem vessels. These are surrounded by a sheath of living cells that control the flow of water into and out of the xylem (Figure 2). Movement of water through and along the wall outside of a cell's plasma membrane is called apoplastic flow. Water can pass along many connected cells via apoplastic flow, for example in the root cortex or leaf mesophyll, bundle sheath extension or epidermis. However there are barriers that interrupt this path in the root endodermis (Steudle, 2000, Zimmermann *et al.*, 2000) and the bundle sheath surrounding veins in the shoot and leaf (Canny, 1988, Canny, 1995, Hachez *et al.*, 2008) (Figure 2). At such points, water needs to move inside the cells to pass the barrier. To enter and exit cells, water must cross the plasma membrane, called the transcellular path. As the plasma membrane is rather impermeable to water, aquaporins provide the channel through which most water moves. It is also possible for water to move between cells while remaining in the cytosol through plasmodesmatal pores between adjacent cells and this is called symplastic flow.

Localisation of PIPs using GUS reporter constructs, mRNA expression or antibodies reveal widespread PIP expression. There is particularly high expression in root tips, the stele of the root and leaf veins (Frangne *et al.*, 2001, Javot *et al.*, 2003, Kaldenhoff *et al.*, 2008, Postaire *et al.*, 2010). The locations surrounding water entry in the xylem where apoplastic flow is blocked are exactly where a high density of aquaporins would be needed to enable transcellular water flow. This simplified scheme for water movement in roots which applies to wheat, has exceptions both between species e.g. lupin (Bramley *et al.*, 2009) and with distance from the root tip (Steudle, 2000). A measure of the ease with which water can flow through roots is hydraulic conductance. This can be determined either by a gradient in hydrostatic pressure or osmotic potential, with the latter reflecting the influence of a plasma membrane barrier (Chaumont & Tyerman, 2014). In wheat, root hydraulic conductivity was rapidly decreased upon exposure to $HgCl_2$ and could be restored with DTT which is consistent with aquaporins controlling water uptake (Clarkson *et al.*, 2000). *Arabidopsis* plants with antisense reductions to PIP1, PIP2 or a combination of these genes were found to have root hydraulic conductance decreased by 50-70% compared to wildtype controls (Martre *et al.*, 2002). In rice, a strong diurnal change in root hydraulic conductance which rises and falls early each day is also seen in the expression of PIP genes (Ishikawa-Sakurai *et al.*, 2014). The transpirational pull of water through the xylem is a consequence of a gradient in water potential between leaves and roots. If transpiration exceeds the ability of resupply from the soil, then there is increased risk of hydraulic failure associated with embolisms forming in the xylem. PIP1s have been found to influence both the vulnerability to embolism as well as the capacity to repair embolism in poplar (Secchi & Zwieniecki, 2014). Thus there are multiple lines of evidence linking aquaporins to the function of water transport in a plant.

In the leaf there are three places where aquaporins are likely to play a significant role in the path of water movement (Figure 2). As with roots, there are multiple solutions to how water moves through leaves (Zwieniecki *et al.*, 2007). A barrier to apoplastic water movement exists at the bundle sheath surrounding the vein which restricts movement to the symplasm and requires transcellular flow for water to enter the mesophyll. Aquaporins surrounding leaf veins have been shown to influence leaf hydraulic conductance in *Arabidopsis* (Prado *et al.*, 2013, Shatil-Cohen *et al.*, 2011). As with roots, diurnal changes in leaf hydraulic conductivity are also correlated to changes in PIP expression in walnut (Cochard *et al.*, 2007) and poplar (Lopez *et al.*, 2013). Flow through the mesophyll can occur either via symplastic, apoplastic or a combination (transcellular) paths and theoretical arguments have been made in support of apoplastic flow dominating the flow of water through the mesophyll (Buckley, 2015). Aquaporins in the plasma membrane of mesophyll cells permit exchange of water between these two pools. When stomatal guard cells mature, their plasmodesmata cease to function (Oparka & Roberts, 2001, Willmer & Sexton, 1979)(Figure 2). Consequently, the hydraulic flow necessary for stomata to open and close requires dynamic control over the permeability of the plasma membrane to water. The function of aquaporins in stomata will be dealt with in more detail subsequently.

Using T-DNA insertion lines of *Arabidopsis*, both PIP1 and PIP2 aquaporins were shown to influence the flux of water into leaves, although it was not possible to separate the contributions from root and shoot (Da Ines *et al.*, 2010). Water leaving leaf veins needs to pass across the plasma membrane to get around the apoplastic barrier to flow at the bundle sheath. Evidence for this comes from plants with miRNA directed at PIP1s. Protoplasts from cells isolated from either the bundle sheath or mesophyll of leaves from constitutively expressed miRNA plants had reduced osmotic water permeability (Sade *et al.*, 2014b). In addition, leaf hydraulic conductance was reduced by the miRNA construct. A second construct was made using the SCARECROW promoter to reduce PIP1 expression specifically within bundle sheath tissue. Intriguingly, this still reduced osmotic water permeability of both bundle sheath and mesophyll protoplasts. However, it is clear that aquaporins are involved in the pathway of water movement through leaves.

Some plant PIPs are CO_2 channels

The diffusion of CO_2 across biological membranes was thought to be non-limiting because of the high solubility of CO_2 in the lipid bilayer. It was surprising therefore, that expression of human AQP1 together with carbonic anhydrase in *Xenopus* oocytes increased their permeability to CO_2 (Nakhoul *et al.*, 1998). Terashima and Ono (2002) reasoned that if aquaporins functioned as a CO_2 channel through the plasma membranes in leaf mesophyll cells, photosynthetic CO_2 uptake should be sensitive to inhibition by $HgCl_2$. Reductions in the rate of CO_2 assimilation at low intercellular CO_2 concentrations following the application of $HgCl_2$ were observed in both *Vicia faba* and *Phaseolus vulgaris* and it was inferred from this that aquaporins increase the permeability of mesophyll cells to CO_2. However, as $HgCl_2$ is a non-specific inhibitor, no precise explanation of these observations was possible. By expressing NtAQP1, a tobacco homologue to human AQP1, in *Xenopus* oocytes, the first demonstration of a plant aquaporin increasing permeability to CO_2 was made (Uehlein *et al.*, 2003). It was argued that improved supply of CO_2 lead to increases in the rate of CO_2 assimilation and subsequent growth of tobacco plants with antisense suppression or overexpression of NtAQP1.

A debate developed over whether aquaporins could indeed enhance membrane permeability to CO_2. Arguments in favour of aquaporins increasing CO_2 permeability (Boron, 2010) and arguments against (Missner *et al.*, 2008a, Missner *et al.*, 2008b, Missner & Pohl, 2009) were clarified in a joint letter (Boron *et al.*, 2011). A CO_2 permeable membrane would not benefit from the inclusion of aquaporins. However, biological membranes in plants are protein rich which reduces the amount of lipid bilayer available for direct diffusion (Kaldenhoff, 2012, Kaldenhoff *et al.*, 2014, Uehlein *et al.*, 2012b). They also have a high sterol content (Uemura *et al.*, 1995) which reduces CO_2 permeability in artificial membranes (Hub & de Groot, 2006, Kai & Kaldenhoff, 2014, Ludewig & Dynowski, 2009, Tsiavaliaris *et al.*, 2015) and biological membranes (Itel *et al.*, 2012). Both of these features suggest that plant plasma membranes may not be very permeable to CO_2 and therefore the inclusion of aquaporins could have an impact on overall permeability (Endeward *et al.*, 2014). There is sufficient functional evidence to justify showing aquaporins enhancing CO_2 diffusion into mesophyll and stomatal guard cells (Figure 2).

By comparison to the numerous examples of PIPs acting as water channels, demonstrations of PIPs enhancing membrane permeability to CO_2 are more limited. This is partly a reflection of the greater difficulty in assaying for CO_2 permeability. One assay method is to isolate membrane vesicles and introduce carbonic anhydrase and a pH sensitive fluorofor into them (Uehlein *et al.*, 2008). The vesicles are then rapidly mixed into a new external solution saturated with CO_2. CO_2 diffusing into the vesicles is converted to bicarbonate by carbonic anhydrase leading to internal acidification which is detected by a change in fluorescence. The derived permeability values appear much less than expected from calculations of mesophyll conductance for intact leaves (Evans *et al.*, 2009). This may reflect the fact that the speed with which CO_2 equilibrates between a new external solution and that inside a vesicle is much faster than the mixing time of stopped flow devices. However, the observed pH change following mixing can be readily fitted with an exponential curve. Concerns about the limitations imposed by unstirred boundary layers (Missner *et al.*, 2008b) have been countered (Endeward *et al.*, 2014, Tsiavaliaris *et al.*, 2015). Alternative methods to detect CO_2 permeability have utilised pH microelectrodes inserted into Xenopus oocytes (Nakhoul *et al.*, 1998), scanning pH near the membrane surface (Uehlein *et al.*, 2012b), expression in yeast cells and following intracellular acidification (Otto *et al.*, 2010) or following the loss of ^{18}O labelled CO_2 from the bathing solution containing cells expressing aquaporins and containing carbonic anhydrase (Itel *et al.*, 2012). Methods for determining permeability currently have an upper limit around 0.1 cm s^{-1} (Itel *et al.*, 2012, Tsiavaliaris *et al.*, 2015). As this is less than the permeability required to account for CO_2 assimilation rates (Evans *et al.*, 2009), it is still a challenge to exactly relate functional performance in assays to that in leaves.

PIPs that have been demonstrated to enhance membrane permeability to CO_2 are shown in red in Table 1. Members of both PIP1 and PIP2 families appear capable of facilitating CO_2 diffusion, but not all are capable. Four cases are shown where expression of a PIP gene did not alter membrane permeability to CO_2. In each of these instances, the PIP had been shown to increase membrane permeability to water thus confirming that it was present as a functional protein in the plasma membrane of either the *Xenopus* oocyte or yeast cell. From the limited number of PIPs where function has been demonstrated unambiguously in *Xenopus* or yeast expression assays, three classes of functional type have been found: water only (PIP2s), CO_2 only (PIP1s), or both water and CO_2 (PIP1s and PIP2s). As already mentioned for water permeability, the expression system influences the function of a given PIP gene. In the case of *Arabidopsis*, the ability to enhance membrane CO_2 permeability when expressed in yeast cells (Heckwolf *et al.*, 2011) was confirmed *in planta* (Uehlein *et al.*, 2012b). However, for NtPIP2;1, no enhancement of CO_2 permeability was detected when expressed in yeast (Otto *et al.*, 2010) and it was only evident when inserted into a CO_2 impermeable triblock copolymer membrane (Uehlein *et al.*, 2012a).

Importantly, when NtAQP1 was expressed in yeast as linked hetero-tetramers with NtPIP2;1, enhancement of CO_2 permeability was most effective when all 4 of the monomers in the tetramers were NtAQP1 (Otto *et al.*, 2010). By contrast, maximum water permeability was achieved with 2 NtPIP2;1 in a tetramer and increasing this to 3 or 4 did not lead to any further increase in permeability to water. These results lead to the suggestion that CO_2 may follow a different path than water through aquaporins. Molecular dynamic simulation has identified that there are three possible routes for CO_2 to cross the tetrameric complex in a membrane (Wang *et al.*, 2007) whereas water moves through the central pore of the monomer (Hub & de Groot, 2006).

Extent of aquaporin influence on mesophyll conductance

It is possible to infer changes to membrane permeability towards CO_2 in leaf mesophyll cells from measurements of mesophyll conductance, the ease with which CO_2 can diffuse between intercellular airspaces and mesophyll chloroplasts. Rice plants transformed with HvPIP2;1 driven by a cauliflower mosaic virus 35S promoter could be separated into two groups (Hanba *et al.*, 2004). In one group, the insertion lead to overexpression of HvPIP2;1 (135%) which was associated with an increase in mesophyll conductance (40%) and increased mesophyll cell wall thickness. In the other group which arose from the same transformation event, transgenic co-suppression lead to a reduction of PIP2;1 (12-33%) and a decrease in mesophyll conductance (15-26%). The changes in mesophyll conductance in relation to the amount of HvPIP2;1 lend *in planta* support to the finding in *Xenopus* oocytes that these PIP proteins influence membrane permeability to CO_2 (Mori *et al.*, 2014). A comparison between a wild type rice and *ospip1;1* rice mutant which knocked out *PIP1;1* but also reduced *PIP1;3*, *PIP2;1* and *PIP2;7* expression, revealed that the mutation decreased stomatal (30%) and mesophyll (50%) conductances which reduced the CO_2 assimilation rate (Ding *et al.*, 2016). This implies that OsPIP1;1 is also likely to alter membrane permeability to CO_2.

In tobacco, the expression of *NtAQP1* was varied by antisense reduction or overexpression to investigate its function *in planta* (Flexas *et al.*, 2006). NtAQP1 content was reduced by 85% or increased two-fold which was associated with a 30% decrease or 34% increase, respectively, in mesophyll conductance in tobacco. When membranes were isolated from the RNAi plants, it was shown that the decrease in NtAQP1 reduced the CO_2 permeability of the chloroplast envelope but did not alter that of the plasma membrane (Uehlein *et al.*, 2008). As was found with rice, expressing a foreign aquaporin gene in tobacco led to an increase in mesophyll conductance (Kawase *et al.*, 2013). However, the expression of the PIP1 type aquaporin from *Mesembryanthemum crystallinum, McMIPB,* increased the rate of CO_2 assimilation by far more than could be expected from simply increasing access to CO_2. Even though the authors observed no change in Rubisco content, an increase in Rubisco activity of nearly 50% would be required to account for the observed increase in CO_2 assimilation rate.

For *Arabidopsis thaliana,* it was found that knocking out *AtPIP1;2* reduced mesophyll conductance by 40% while knocking out *AtPIP2;3* did not alter mesophyll conductance relative to wildtype (Heckwolf *et al.*, 2011). The reduction in mesophyll conductance increased the draw-down in CO_2 partial pressure from the intercellular airspaces to the sites of carboxylation (C_i-C_c) from 83 to 102 µbar. These results were consistent with the effects on CO_2 and water permeability they observed when the two genes were expressed in yeast. However, two other reports with *Arabidopsis* using miRNA suppression of PIPs or knockout and overexpression of *PIP1;4* are less clear. PIP1s were suppressed to slightly different degrees in two lines using miRNA and reductions in mesophyll conductance of 10 and 20% were presented (Sade *et al.*, 2014b). The veracity of these mesophyll conductance estimates seems unlikely as the mesophyll drawdown (C_i-C_c) that can be calculated (200 µbar) is double that normally seen. In the other paper where *PIP1;4* was altered (Li *et al.*, 2015b), the changes reported for CO_2 assimilation rate greatly exceed that expected from the reported change in mesophyll conductance. The results imply that a large increase or decrease in Rubisco activity accompanied the changes in mesophyll conductance. Another concern is apparent in the decline in mesophyll drawdown for the *Atpip1;4* plants from 64 to 43µbar when it should have increased to 90 µbar.

When RNAi against *PIP1* was engineered into *Populus tremula x alba*, large reductions in *PIP1;1* and *PIP1;3* were observed with no change to *PIP2* genes (Secchi & Zwieniecki, 2013). While mesophyll conductance was reported to have decreased by 50%, the mesophyll drawdown for the wild type can be calculated to be only 10 µbar. This is highly unlikely and means that the magnitude of the change in mesophyll conductance is probably very different. In another experiment with *Populus,* RNAi was used to reduce *PIP* expression (Bi *et al.*, 2015). Although both lines had similar reductions in several PIP1 and PIP2 proteins, the phenotypes differed with mesophyll conductance increasing in one and not changing in the other compared to wild type controls. Despite this inconsistent pattern, the authors drew attention to the increase in mesophyll conductance.

Unfortunately, for several of the papers mentioned above implying causal links between changes in aquaporins and mesophyll conductance, there are serious doubts. Mesophyll conductance is a complex trait that depends upon the surface area of chloroplasts adjacent to intercellular airspace per unit leaf area, cell wall thickness and membrane permeability (Evans *et al.*, 2009). To assess what might be expected from altering PIP expression, we have modelled the effect of changing membrane permeability on photosynthetic characteristics by assuming that altered *PIP* expression only changes mesophyll conductance (Figure 3). Two scenarios are illustrated where either stomatal conductance or intercellular CO$_2$ partial pressure is held constant as mesophyll conductance varies. For these curves, the normal leaf is assumed to have a Rubisco activity of 100 µmol m^{-2} s^{-1} and a mesophyll conductance of 0.3 mol m^{-2} s^{-1} bar^{-1} which results in a mesophyll drawdown of 72 µbar under ambient CO$_2$ conditions. Decreasing mesophyll conductance results in a reduction in CO$_2$ assimilation rate and an increase in mesophyll drawdown (C$_i$-C$_c$) or vice versa if mesophyll conductance increases (Figure 3a,b). Data from three papers are added in Figure 3b to illustrate when it is consistent with expectations (Flexas *et al.*, 2006, Hanba *et al.*, 2004) or when the mesophyll conductance information seems unreliable (Li *et al.*, 2015b).

Short term responses to temperature

Temperature directly affects the permeability of membranes. In the short term this reflects the properties of the lipids and proteins that comprise the membrane. Over the longer term, the composition can be altered which can adjust the response of membrane permeability to temperature. The activation energy from the Arrhenius equation, E$_a$, is useful for describing how responsive membrane permeability is to a change in temperature. E$_a$ values associated with diffusion through the lipid bilayer are high whereas those through aquaporins are low, and is similar to that associated with simply the viscosity of water. Consequently, the activation energy provides an indication of the relative influence of these two pathways (lipid bilayer and pores) to the overall diffusion across a membrane.

Additional information discriminating between pathways is obtained by assaying permeability in the presence or absence of transporter inhibitors. For red blood cells, permeability to water was greatly reduced by the inhibitor p-chloromercuribenzoate while concomitantly leading to an increase in E_a from 20 to 60 kJ mol^{-1} (Brahm, 1982). When tonoplast membranes were compared against plasma membranes, it was found that tonoplast membranes were much more permeable to water and had much lower E_a than plasma membranes (10.5 versus 57 for tobacco, 23 versus 48 kJ mol^{-1} for wheat (Maurel *et al.*, 1997, Niemietz & Tyerman, 1997). Tonoplast membrane permeability could be inhibited by mercury indicating that aquaporins normally contributed substantially to the overall flux of water across the membrane. By contrast, plasma membrane permeability was insensitive to mercury. However, in the case of plasma membranes isolated from wheat roots, a ratio of osmotic to diffusive permeability of 3 indicated that there must have been mercury insensitive aquaporins still contributing to the flow of water (Niemietz & Tyerman, 1997). Over-expression of *AtPIP1;4* or *AtPIP2;5* genes in Arabidopsis (Lee *et al.*, 2012) and *PtdPIP2;5* in poplar (Ranganathan *et al.*, 2016) dramatically reduced the E_a for root hydraulic conductivity without affecting the value at 20°C. Sensitivity to mercury inhibition was also reduced for Arabidopsis over expressing these PIP genes (Lee *et al.*, 2012), consistent with the properties of plasma membranes isolated from wheat roots.

Aquaporins can be switched in a gating mechanism that responds to divalent cations such as Ca^{2+} or low pH which reduces membrane permeability to water and subsequently increases E_a (Gerbeau *et al.*, 2002, Tournaire-Roux *et al.*, 2003). Gating complicates the interpretation of permeability assays because closure of aquaporins during membrane isolation could lead to an underestimation of membrane permeability. It is also possible that gating changes with temperature which could confound the estimation of E_a, particularly in assays involving whole tissue rather than isolated membranes, such as root hydraulic conductivity.

There is limited experimental data available for the temperature response of membrane permeability to CO_2. For plasma membranes isolated from pea leaves, E_a varied between 32-36 kJ mol^{-1} in the presence of sufficient carbonic anhydrase to catalyse the detection system (carbonic anhydrase itself has an E_a of 51.5 kJ mol^{-1}) (Zhao *et al.*, 2016). The addition of either silver sulfadiazine (a potent inhibitor of water permeability (Niemietz & Tyerman, 2002) or 4, 4'-diisothiocyano-2, 2'-stilbenedisulfonic acid (which inhibits CO_2 permeability associated with human AQP1 (Endeward *et al.*, 2006)) reduced permeability to water but did not affect permeability to CO_2 of plasma membranes isolated from pea leaves.

The temperature response of mesophyll conductance to CO_2 in part reflects changing membrane permeability. When Bernacchi et al. (2002) observed a strong temperature dependence of mesophyll conductance, they suggested that as this did not conform to what was expected by simple diffusion, it was likely that an enzyme or protein-facilitated process was involved. Subsequently a simple model for the temperature response was proposed which considered liquid diffusion and membrane permeability as the two components (Evans & von Caemmerer, 2013). To account for variation in temperature responses of mesophyll conductance between species in the model, both the liquid path-length and activation energies had to be varied between species (E_a 36 - 76 kJ mol^{-1} (von Caemmerer & Evans, 2015). However, since carbonic anhydrase facilitates CO_2 diffusion within mesophyll cells, it is also possible that the apparent E_a represents a combination of changes in membrane permeability and carbonic anhydrase activity. There is an obvious need to compare the temperature response of mesophyll conductance in a manner analogous to the inhibitor studies for water permeability. Since inhibitor specificity is problematic with intact plant tissue, a practical alternative would be to compare wildtype plants against plants where aquaporin expression had been either increased or reduced. A reduction in CO_2 permeable aquaporin contribution should increase the apparent activation energy of mesophyll conductance, whereas an increase in CO_2 permeable aquaporin activity should decrease the activation energy values.

Role of CO_2 permeable aquaporins in C_4 photosynthesis

Despite increasing research on CO_2 permeable aquaporins in C_3 photosynthetic plants, their role in CO_2 diffusion in C_4 plants is largely unknown (von Caemmerer & Furbank, 2016). Given the high rates of CO_2 fixation characteristic of the C_4 pathway, a large mesophyll conductance is needed to miminise the drawdown between intercellular airspaces and the cytosol of mesophyll cells where carbonic anhydrase catalyses the conversion of CO_2 into bicarbonate, the substrate for PEP carboxylase. Consequently, attention should be focussed on aquaporins in the plasma membrane of mesophyll cells. The absence of Rubisco from mesophyll chloroplasts may mean that CO_2 permeable aquaporins are not needed in the chloroplast envelope membranes. A comparison between the proteomes of chloroplast envelopes from bundle sheath and mesophyll chloroplasts in maize revealed only one aquaporin (Majeran *et al.*, 2008). PIP2;4 was present in bundle sheath but not mesophyll chloroplasts, but as yet there is no evidence indicating whether ZmPIP2;4 enhances permeability to CO_2 and/or water. The specialised Kranz anatomy of C_4 plants, where mesophyll cells are tightly packed around bundle sheath cells, means there is limited surface area available for direct CO_2 diffusion between intercellular airspace and bundle sheath cells (von Caemmerer *et al.*, 2007). However, a high permeability of the plasma membrane of bundle sheath cells would be undesirable as it could increase the escape of CO_2 from the bundle sheath and reduce the efficiency of the C_4 pump. Analysis of a leaf transcriptome comparison between two *Cleome* species with C_3 or C_4 photosynthesis revealed a 20 fold increase in the abundance of an mRNA coding for a PIP1;2 homolog in the C_4 plant (Braeutigam *et al.*, 2011). A homolog of this gene in maize (ZmPIP1;2, Table 1) has been shown to increase water permeability (Bienert *et al.*, 2012, Fetter *et al.*, 2004) while its homolog in *Arabidopsis* (AtPIP1;2) increases permeability to CO_2 (Heckwolf *et al.*, 2011). It remains to be shown whether this PIP increases permeability to CO_2 in C_4 species.

The number of PIPs in C_4 species are comparable to their C_3 counterparts (see Figure 1): 14 PIPs in *Sorghum bicolor* (Reddy *et al.*, 2015) and 13 PIPs in *Zea mays* (Chaumont *et al.*, 2001). Within C_4 plants, only 2 PIPs (ZmPIP1;5 and ZmPIP1;6) have been identified as CO_2 permeable (Heinen *et al.*, 2014). When co-expressed with a PIP2, both also conferred water permeability. Interestingly, these PIPs had significantly higher expression in the leaf epidermis suggesting a role in stomatal complexes (Heinen *et al.*, 2014). A key area for future studies is identifying which PIPs are permeable to CO_2 in C_4 plants and examining their effects on mesophyll CO_2 conductance.

Regulation of aquaporin expression

Aquaporins have varied transcriptional regulation. Their expression has been shown to be tissue specific and strongly influenced by environmental factors including drought (Alexandersson *et al.*, 2010, Alexandersson *et al.*, 2005, Jang *et al.*, 2004, Smart *et al.*, 2001), salinity (Qian *et al.*, 2015, Zhu *et al.*, 2005), temperature (Lee *et al.*, 2012, Ranganathan *et al.*, 2016) and humidity (Laur & Hacke, 2013) as well as diurnal (Lopez *et al.*, 2003, Moshelion *et al.*, 2002) and circadian clock (Harmer *et al.*, 2000, Takase *et al.*, 2011) regulation.

Expression studies have revealed developmental and tissue specific profiles of aquaporin abundance in leaves and roots. Changes in aquaporin abundance over leaf developmental gradients have been observed in maize (Hachez *et al.*, 2008) and barley (Besse *et al.*, 2011) with expression highest in the elongating zone of the leaf. Detailed cell specific localisation of rice aquaporins with different water transport activities implies there are distinct roles for each aquaporin within the rice root (Grondin *et al.*, 2016, Sakurai *et al.*, 2008). The preferential expression profile of individual aquaporins within the leaf or root organ may provide a clue towards their putative role. For instance in the *Arabidopsis* leaf, *AtPIP1;2* has high gene expression in mesophyll cells and has been shown to enhance membrane permeability to CO_2

(Heckwolf *et al.*, 2011) whereas PIPs expressed solely in the leaf vasculature (e.g. *AtPIP2;1*, *AtPIP2;6*) are more likely to be involved in water permeability (Prado *et al.*, 2013). Aquaporins may also have dual roles as several have been found to influence membrane permeability towards both water and CO_2 (Table 1). The actual role may also depend on the composition of heterotetramers (Berny *et al.*, 2016, Otto *et al.*, 2010).

In response to drought stress, plants close their stomata to reduce transpiration and down regulate aquaporin gene expression. As a consequence of stomatal closure, intercellular CO_2 partial pressure (C_i) decreases which also reduces photosynthesis. Whilst the majority of PIPs are down regulated during drought stress certain PIP isoforms are upregulated. This could increase mesophyll conductance to compensate for the declining availability of CO_2 in the intercellular airspaces. Interestingly, AtPIP1;4 which increases membrane CO_2 permeability (Li *et al.*, 2015b), was one of only a few aquaporins to be consistently upregulated in drought stress experiments in *Arabidopsis* (Alexandersson *et al.*, 2010, Alexandersson *et al.*, 2005, Jang *et al.*, 2004). Similarly in tobacco, while the water permeable aquaporins NtPIP1;1 and NtPIP2;1 were down regulated in response to drought, the CO_2 permeable but weakly water permeable NtAQP1 was upregulated (Mahdieh *et al.*, 2008). In *Nicotiana plumbaginifolia*, ABA induced by drought or applied to a detached leaf decreased both stomata and mesophyll conductances but whether the mechanism(s) involved aquaporins or carbonic anhydrase remains to be demonstrated (Mizokami *et al.*, 2015).

Distinct aquaporin transcript responses to changes in CO_2 concentrations have been observed in tobacco leaves (Secchi *et al.*, 2016). Growth in low atmospheric CO_2 concentrations increased the expression of the CO_2 permeable NtAQP1, whereas the water permeable NtPIP2;1 did not change. This fits with the argument that CO_2 permeable aquaporins are upregulated under drought in order to maintain photosynthetic rates when CO_2 becomes limiting due to stomatal closure. Since different PIP genes confer different functions, it could be possible to reduce water permeability in response to drought by down regulating one gene while increasing CO_2 permeability by upregulating another.

Aquaporin protein structure and trafficking

The aquaporin protein forms a helical bundle consisting of six membrane spanning domains (H1 to H6) connected by five loops (LA to LE). LA, LC and LE reside on the apoplastic side of the membrane, while LB and LD along with both terminal tails are exposed to the cytoplasm (Tornroth-Horsefield *et al.*, 2006)(Figure [illegible]). LB and LE each form half helices that insert into the membrane and meet in the middle to form the pore. Situated at the meeting point are two highly conserved NPA domains that together with four residues located on the apoplastic sides of H2 (F81) and H5 (H210) and within LE (T, R) (referred to as the ar/R filter) govern to a large extent the substrate specificity of the pore.

Aquaporin monomers form homo- and hetero-dimers and subsequently tetramers in the ER before being transported and integrated into the membrane. Subcellular trafficking of aquaporins to their respective membranes is complex and represents another significant control over membrane permeability by controlling channel density within the membrane. Several recent reviews detail aquaporin trafficking in plants (Chaumont & Tyerman, 2014, Chevalier & Chaumont, 2015, Hachez *et al.*, 2013, Luu & Maurel, 2013). Briefly, the majority of our understanding of aquaporin trafficking has been obtained by studying Arabidopsis and maize PIPs. Maize PIPs have a distinctive feature whereby ZmPIP2s can reach the plasma membrane when expressed alone while ZmPIP1s are retained in the ER and require hetero-oligomerisation with ZmPIP2s to exit the ER. This distinction is observed both in plants and heterologous expression systems in oocytes and yeast (Bienert *et al.*, 2014, Fetter *et al.*, 2004, Zelazny *et al.*, 2007). Similar relationships between PIP1s and PIP2s also occur in other species (Jozefkowicz *et al.*, 2016, Mahdieh *et al.*, 2008, Mori *et al.*, 2014, Yaneff *et al.*, 2014). However, this relationship is not absolute as some PIP1s appear capable of reaching the plasma membrane alone as determined directly by PIP fusions with fluorescent proteins or implicitly via increased membrane permeability when expressed in oocytes or yeast (Berny *et al.*, 2016, Ding *et al.*, 2004, Fitzpatrick & Reid, 2009, Heckwolf *et al.*, 2011, Mosa *et al.*, 2016, Navarro-Rodenas *et al.*, 2013, Otto *et al.*, 2010, Sabir *et al.*, 2014, Zhang *et al.*, 2010).

Several Arabidopsis and maize PIP2 isoforms contain a so-called diacidic motif, more precisely a DxE configuration (X; underdetermined), in the N-terminal region which facilitates exiting from the ER into the secretory pathway (Sorieul *et al.*, 2011, Zelazny *et al.*, 2009). Additionally, a LxxxA motif in the third transmembrane domain, which is highly conserved among PIP2 proteins, also assists exit out of the ER and correct sorting in the Golgi (Chevalier *et al.*, 2014). However, both motifs alone or in combination are incapable of translocating a ZmPIP1 to the plasma membrane, indicating the existence of other yet to be identified export or retention signals. More recently, a F220A mutation within the fifth transmembrane domain of ZmPIP1;2 was found to effectively increase ZmPIP1;2 localisation to the membrane of oocytes and subsequently increase water permeability (Berny *et al.*, 2016). Post-Golgi transport and insertion of PIPs into the plasma membrane involve SNARE (soluble *N*-ethylmaleimide-sensitive factor protein attachment protein receptor) proteins, which are well known vesicle traffickers in eukaryotes. Two SNARE proteins in SYNTAXIN OF PLANTS 121 and 67 (SYP121 and SYP67) have been shown to physically interact and ensure proper delivery of AtPIP2;7 and ZmPIP2;5 into the plasma membrane (Besserer *et al.*, 2012, Hachez *et al.*, 2014). It is speculated that different SNARE isoforms may control subcellular routing of PIPs and possibly more broadly aquaporins at different stages of delivery to respective membranes (Hachez *et al.*, 2013).

Once in the membrane, each aquaporin monomer of the tetramer complex constitutes an independent pore, the activity of which is determined by its amino acid composition, interactions with accompanying monomers, post-translational modification and interactions with signalling molecules and other proteins (detailed below). Aquaporins appear to be continually cycled in and out of membranes which may aid in maintaining a homogeneous distribution within the membranes. There are also PIPs and NIPs that are directionally distributed within the plasma membrane of leaf and root cells, but the mechanism and its physiological implication is unknown (Chevalier & Chaumont, 2015). In addition to resting state cycling, aquaporins can also be rapidly removed from their respective membranes in response to osmotic and salt stress subsequently reducing membrane water permeability (Boursiac *et al.*, 2005, Dhonukshe *et al.*, 2007, Li *et al.*, 2011). This rapid response is partly governed by the phosphorylation status of the C-terminal tail (Prak *et al.*, 2008).

Gating

Through conformational changes of the tertiary protein structure, membrane channels can switch between open and closed states (gating). Gating is a general mechanism of many types of membrane channels for controlling permeability (Verma *et al.*, 2015b). Three dimensional structures of about a dozen unique aquaporin proteins from species across the major phylogenetic kingdoms have facilitated our understanding of aquaporin gating (Gonen & Walz, 2006, Kreida & Tornroth-Horsefield, 2015, Sachdeva & Singh, 2014, Verma *et al.*, 2015a). Differences exist in the structural moieties and residue topology involved in gating between these divergent aquaporins (Kreida & Tornroth-Horsefield, 2015, Sachdeva & Singh, 2014). Several resolved structures of spinach PIP2;1 (SoPIP2;1) in an open and closed state, phosphorylated mutant forms and at high and low pH (pH 6 vs. pH8), together with molecular dynamic simulations have been instrumental in understanding plant aquaporin gating (Frick *et al.*, 2013, Khandelia *et al.*, 2009, Kukulski *et al.*, 2005, Nyblom *et al.*, 2009, Tornroth-Horsefield *et al.*, 2006).

Inferred from these structural studies is that gating is in a dynamic equilibrium mediated through different combinations of interactions in response to changing environmental cues. The physical blockage of PIPs occurs through capping of the cytosolic entrance by loop D, causing insertion of a hydrophobic leucine residue into the channel entrance (L197, Figure 4). Anchoring of loop D in a closed state is accomplished through interactions between residues of loop D and residues in the N-terminal tail, loop B, transmembrane helix 2 (H2) and C-terminal tail (Figure 4; Supplementary Figure 1). Disruption of these interactions displaces loop D resulting in removal of L197 and opening of the channel. Phosphorylation, divalent cations (Ca^{2+}) and cytosolic pH mediate the interactions between the open and closed positioning of loop D, and subsequently affect membrane permeability (Supplementary Figure 1; Supplementary Table 1) (Di Pietro *et al.*, 2013, Fischer & Kaldenhoff, 2008, Gerbeau *et al.*, 2002, Grondin *et al.*, 2015, Johansson *et al.*, 1998, Nyblom *et al.*, 2009, Tournaire-Roux *et al.*, 2003, Verdoucq *et al.*, 2008). Aquaporins gate shut in response to elevated cytosolic Ca^{2+} concentrations and lower cytosolic pH. Such conditions are commonly observed under flooding stress, mechanical stimulation, pathogen attack and ROS accumulation (Chaumont & Tyerman, 2014, Monshausen & Gilroy, 2009, Monshausen & Haswell, 2013, Tournaire-Roux *et al.*, 2003). Ca^{2+} triggers pore closure by ligating to N28 and G30 inducing a series of interactions, involving loop B, that draws loop D into a closed configuration (Figure 4; Supplementary Figure 1). Low pH causes channel closure through protonation of H193. In the absence of a bound cation, the protonated H193 (denoted as $H193^{+}$) draws loop D into a closed state through interaction with loop B. In the presence of a bound cation, the repositioned N-terminal tail allows $H193^{+}$ to bind D28 (Supplementary Figure 1). These networks of interactions are further governed by three cytoplasmically exposed serine residues that are targets for phosphorylation (S115, S188 and S274) (Di Pietro *et al.*, 2013, Hsu *et al.*, 2009, Kline *et al.*, 2010, Nyblom *et al.*, 2009). In an unphosphorylated state, such as drought conditions (Kline *et al.*, 2010), these serine residues stabilise the closed loop D structure through interactions with the N-terminal tail (S115), within loop D (S188) and by creating a steric hindrance for the open loop position (S274) (Figure 4; Supplementary Figure 1). Phosphorylation on the other hand (symbolised with -PO_4), swings loop D away from the channel entrance by repulsion forces that dislodge Ca^{2+} from E31 (S115-PO_4), promote interactions between loop D and the C-terminal tail (S188-PO_4) and provide room for the open state repositioning of loop D (S274-PO_4).

Complementing the structural analysis is functional testing of aquaporin gating using mimetic mutations (Supplementary Table 1). Mimetic mutants do not necessarily exert the same level of effect as the modelled response (Nyblom *et al.*, 2009) and can show variability between experiments. This could be due to discrepancies in the properties of the mimetic residues, inherent variation between expression systems, or the fact that some components of the network exert an overriding influence over other components (e.g. $H193^{+}$ > S-PO_4 (Frick *et al.*, 2013, Nyblom *et al.*, 2009). Interestingly, the characteristics of pH gating are present in PIPs across the entire plant kingdom, whereas motifs associated with Ca^{2+} gating appear specific to land plants (Anderberg *et al.*, 2011). The evolution of a more intricate gating mechanism likely reflects the need for greater control over hydraulic and other substrate transport in a terrestrial environment.

Beyond gating

Beyond gating, the analysis of APQ tertiary structures and mutagenic approaches have also identified residues mediating channel activity. These have been summarised in Figure 4 and Supplementary Table 2 (for consistency all references of residue positions are in accordance to SoPIP2;1 in Figure 4; positioning of the equivalent residue within the actual tested aquaporins are listed in Supplementary Table 2). Substitutions at some of these positions (W79, F86 and G97) cause a complete loss in channel activity in multiple PIP variants, indicating a core role in channel integrity. Others, such as position 87, are variable between PIP1s and PIP2s and contribute to the differences in water permeability between these groups in rice (Zhang *et al.*, 2010). Loop E, which forms the apoplastic entrance half of the channel, has a number of important positions. In addition to the NPA motif and half of the ar/R filter, three other residues influence water transport activity (Suga & Maeshima, 2004). Modelling of G228A mutations in loop E have predicted a potential doubling of water permeability. Although this was modelled with mammalian AQP1, G228 shows a high degree of conservation and the inference may be applicable to plant aquaporins. So far at least one-third of the residues in loop E have been identified as influencing permeability.

In addition to pore lining residues, the functional relevance of a number of transmembrane helix residues have recently been elucidated. Sequence conservation of 1500 MIPs across the major kingdoms has identified up to 40 positions within the transmembrane helices that show a high degree (avg. 92%) of conservation (Verma *et al.*, 2015b). Two-thirds of these positions are occupied by small and weakly polar residues that are speculated to facilitate condensed helical packing, allowing for a tight union between interacting transmembrane helices. Larger predominantly bulky hydrophobic residues have been identified, using alanine scanning combined with structure based modelling, as facilitating intra-monomer and inter-monomer interactions that establish and stabilise tetramer formation and subsequently functionality of the *Arabidopsis* PIP2;1 homo-tetramer (Yoo *et al.*, 2016). Structural modelling combined with mutagenesis was used to identify residues important in water channel activity, subcellular localization, protein abundance, and physical interaction between maize PIP1;2 and PIP2;5 (Berny *et al.*, 2016).

Interestingly, certain mutant forms can increase water activity in one monomer while hindering activity of the adjacent monomer. Together, these two reports extend on previous knowledge (Bienert *et al.*, 2012, Fetter *et al.*, 2004, Heinen *et al.*, 2014, Jozefkowicz *et al.*, 2013, Otto *et al.*, 2010) of the importance and interplay of individual monomers to the collective functionality of the tetrameric complex. Beyond the transmembrane helices, residues in loop A are important in tetrameric organisation. C69 of each monomer interacts to stabilise the tetramer (Bienert *et al.*, 2012, Kukulski *et al.*, 2005), while residues 64 and 65 are implicated in influencing interactions between PIP1 and PIP2 of *Beta vulgaris* (Jozefkowicz *et al.*, 2013).

Most of these studies have examined effects on water permeability. Single amino acid substitutions (e.g. SoPIP2;1 positions G97W, S115A and H193K; Supplementary Tables 1 and 2) have been shown to block CO_2 and H_2O_2 in conjunction with water transport through PIPs (Bienert *et al.*, 2014, Shelden *et al.*, 2009, Wang *et al.*, 2016). But these induce channel closure and are therefore general in effect. However, a single amino acid change has been found that confers different CO_2 permeability between HvPIP2;3 and HvPIP2;4 (Mori *et al.*, 2014). Each of the 6 amino acids that differed between these two aquaporins was mutated, but only the I to M switch at the apoplastic end of helix 6 affected CO_2 permeability (Figure 4). Having I opposed to M conferred CO_2 permeability in both HvPIP2;3 and HvPIP2;4. However, both aquaporins remained permeable to water regardless of which two amino acids were present.

Interactions with other proteins

Besides interactions amongst each other, aquaporins also form protein-protein interactions with other protein types (reviewed in (Maurel *et al.*, 2015, Sjohamn & Hedfalk, 2014)). Many of these are involved in trafficking of aquaporins to and from membranes (Chevalier & Chaumont, 2015). But evidence is emerging of interactions that directly influence transport efficiency when membrane bound. AtPIP2;1 has been recently shown to interact with the carbonic anhydrase enzyme, βCA4, as part of a transport metabolon regulating stomatal closure in response to internal leaf CO_2 concentrations (Wang *et al.*, 2016). Although not established, this interaction likely occurs via the cytoplasmic located C-terminal tail of AtPIP2;1, as has been shown for mammalian AQP1 and CARBONIC ANHYDRASE II (CAII) (Vilas *et al.*, 2015). The C-terminal tail of AQPs appears a common site for interactions with non-aquaporin cytosolic partners (Masalkar *et al.*, 2010, Sjohamn & Hedfalk, 2014). Since CAs catalyse the conversion of CO_2 and water into bicarbonate and hydrogen ions, we can speculate that the coupling of AtPIP2;1 and βCA4 could enhance transport by depleting CO_2 and creating a concentration gradient immediately adjacent to the pore. A similar process is proposed to account for the enhanced water flux through mammalian AQP1 when associated with CAII (Vilas *et al.*, 2015). CAII is also connected with the Rh CO_2 transporter as part of a CO_2 metabolon in the erythrocyte membrane (discussed in (Kustu & Inwood, 2006)). CAII interacts and enhances the activity of numerous mammalian membrane transporters (Becker & Deitmer, 2007, Becker & Deitmer, 2008, Becker *et al.*, 2010, Krishnan *et al.*, 2015, Li *et al.*, 2006, Pushkin *et al.*, 2004), suggesting a need to further explore plant PIP-CA interactions in modulating both water and CO_2 transport. An analogous AQP-involved metabolon occurs with the interaction between the ammonia transporter Nodulin 26 (a NIP aquaporin) and Glutamine Synthase, the catalytic substrate of which is ammonia (Masalkar *et al.*, 2010).

Since aquaporins span the membrane, interactions with non-aquaporin partners are not limited to cytosolic side. Recently, AtPIP1;4 has been reported to interact with the plant pathogen bacterial protein Harpin 1 (Hpa1) on the apoplastic side of the plasma membrane (Li *et al.*, 2015b). This interaction was associated with enhancement in plant growth upon the exogenous treatment with Hpa1. The PM localised AtPIP1;4-Hpa1 interaction increases the activity of AtPIP1;4 in mediating transmembrane CO_2 transport and leaf photosynthesis. This result extends the biological role of aquaporin beyond simply transport and into sensing biotic signals (Li *et al.*, 2015b).

Aquaporins and stomatal function

Stomata are the gatekeepers for water and CO_2 diffusing between the atmosphere and the inside of the leaf. Guard cells respond to many signals and can change the aperture of the pore by swelling and shrinking to open or close (Kim *et al.*, 2010). Since mature guard cells have non-functional plasmodesmata (Oparka & Roberts, 2001), the flux of water into and out of guard cells depends upon aquaporins. Detailed analysis of the expression pattern for aquaporin genes has been made for maize guard cells (Heinen *et al.*, 2014). Each of the 6 genes for PIP1 and PIP2 were expressed with PIP1;1 (66% of total PIP transcripts) and PIP1;3 (12%) being the most abundant. Five PIPs (PIP1;4 and PIP2;3-6) were in low abundance. Expression of all of the abundant PIP genes was found to show strong diurnal variation. ZmPIP1;5 and ZmPIP1;6 which each represented about 2.5% of the total PIP expression, were shown to enhance water and CO_2 permeability when expressed in *Xenopus* oocytes or yeast, respectively.

Two recent discoveries have revealed how aquaporins fit into the stomatal signalling response pathway network. For *Arabidopsis* plants lacking AtPIP2;1, ABA is unable to trigger stomatal closure. This pathway involves Open Stomata 1 protein kinase which has been shown to phosphorylate AtPIP2;1 at S121

(S115 in Figure 4) which increases membrane permeability to water (Grondin *et al.*, 2015). AtPIP2;1 has also been shown to increase CO_2 permeability of *Xenopus* oocytes and interacts with the carbonic anhydrase βCA4 (Wang *et al.*, 2016). Four independent methods were used to demonstrate interactions between βCA4 and AtPIP2;1 in plasma membranes. When co-expressed with a βCA4, a slow anion channel (SLAC1) and a Ca^{2+}-dependent protein kinase (CPK6 or CPK23), AtPIP2;1 conferred CO_2 sensitivity to SLAC anion channel activity. Introducing a single point mutation G103W disrupted the ability of AtPIP2;1 to increase CO_2 permeability (Wang *et al.*, 2016). Thus the presence of AtPIP2;1 in the plasma membrane of guard cells enables the sensing of external CO_2 and this or ABA can trigger phosphorylation at S121 which enhances permeability to water in association with SLAC1 activity to drive stomatal closure.

Engineering aquaporins to improve plant performance

Abiotic stress, such as drought, salinity and extreme temperature, can reduce the average yield of major crops by more than 50 % (Wang *et al.*, 2003). The dynamic nature of aquaporins means they can adjust their transcript level in response to drought and other abiotic stresses (Alexandersson *et al.*, 2005, Moshelion *et al.*, 2015). Their role in response to different environmental stresses has been reviewed comprehensively by Afzal and colleagues (2016). Here we have summarised research which has modified aquaporin expression with the aim of engineering plants with improved growth and tolerance to different abiotic stresses (Table 3).

Depending on the desired outcome, some researchers have selected an aquaporin from a species which is naturally tolerant to a given stress and transferred it to another species. For instance, AcPIP2 (Li *et al.*, 2015b) from the saltbush *Atriplex canescens* was used for salt tolerance and FaPIP2;1 (Zhuang *et al.*, 2015) from the grass *Festuca arundinacea* and JcPIP2;7 or JcTIP1;3 from *Jatropha curcas* (Khan *et al.*, 2015) were used for drought tolerance. Others have used an aquaporin with a known permeability function to engineer an improved plant. Overexpression of *NtAQP1* has resulted in enhanced plant performance under normal as well as salt stress conditions in a range of plants including *Arabidopsis* (Sade *et al.*, 2014a), tomato (Kelly *et al.*, 2014, Sade *et al.*, 2010) and tobacco (Uehlein *et al.*, 2003). Specifically, increases in biomass and yield, photosynthetic rate, mesophyll and stomatal conductance and root hydraulic conductivity have been observed when NtAQP1 expression was manipulated.

Significant improvements in plant growth in banana have been found when overexpressing either the native *MusaPIP1;2* or *MusaPIP2;6* over a range of stress conditions including drought, salt and cold treatment (Sreedharan *et al.*, 2013, Sreedharan *et al.*, 2015). Overexpression of endogenous aquaporin genes have also been shown to improve plant growth in a range of species including *SlTIP2;2* in *Solanum lycopersicum* under drought and salt stress (Sade *et al.*, 2009) and improved tolerance to boron when the native *AtTIP5;1* was overexpressed in *Arabidopsis* (Pang *et al.*, 2010). Benefits to plant performance also extends to improved immunity with a recent report showing that overexpression of *AtPIP1;4* enhances resistance of Arabidopsis to infection by the biotrophic bacterial pathogen, Pseudomonas syringae (Tian *et al.*, 2016). However, broad improvement to stress tolerance is uncommon and generally increasing expression of one aquaporin to improve tolerance to one stress has a detrimental effect under a different stress. This is the case for a range of *Arabidopsis* PIPs where overexpression has resulted in improved growth under normal conditions (Aharon *et al.*, 2003) or cold stress (Jang *et al.*, 2007) but increased the plant's susceptibility to drought.

Engineering better plants by modifying aquaporin expression is a complex and difficult task but expression can be made tissue specific (Sade *et al.*, 2014a). Monomer function and trafficking could also be modified depending on the heterodimerisation with different PIPs (Berny *et al.*, 2016, Fetter *et al.*, 2004). If mesophyll conductance could be increased, there is potential to improve both photosynthetic rate and transpiration efficiency (Figure 3). The improvement in transpiration efficiency would depend on feedback controls in the leaf that could either keep stomatal conductance constant (which would mean transpiration efficiency would increase as mesophyll conductance increased), or intercellular CO_2 partial pressure constant (there would be no change in transpiration efficiency). Stomatal guard cell functioning could also be targeted to alter sensitivity to ABA or CO_2, or influence the time constant for changing aperture. Any of these would impact on transpiration and transpiration efficiency.

Conclusions

PIP aquaporin diversity exists at many levels and the functional roles of many of the PIP genes still remain to be demonstrated. There are more PIP genes known to affect membrane permeability to water than CO_2 and this limits our ability to identify amino acids that confer substrate specificity. The dynamic changes in gene expression and abundance of tetramers in the plasma membrane, together with the rapid regulation through gating provide plant cells with highly responsive mechanisms to control the flux of water into cells and through the plant. Despite the challenge of assaying for CO_2 permeability, there is a need to characterise many more of the PIP genes for their effect on CO_2 permeability, particularly in C_4 species. Functional screens should also look at other substrates to enhance the possibility of revealing key motifs. There is also a need to consider what other molecules interact with the PIP aquaporins as they are ideally placed to act as sensors. How plants gain and use both water and CO_2 is crucial for plant productivity and growth under stressful conditions, so aquaporins are sensible targets when trying to increase our mechanistic understanding or seeking ways to improve plants.

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Table 1. Functional properties of PIP1 and PIP2 aquaporins. Aquaporins conferring increased permeability to water (cells highlighted in blue) when expressed in *Xenopus* oocytes are marked as strong (✓), weak (w), or requires co-expression with another PIP (+, see table 2). Aquaporins conferring increased permeability to water when expressed in yeast are marked as (Y), or (×) if expression in yeast increased CO_2 permeability but did not alter water permeability. Aquaporins conferring increased permeability to CO_2 (cells highlighted in red) when expressed in *Xenopus* oocytes (✓) or when expressed in yeast (Y) or incorporated into a triblock copolymer membrane (P). Instances marked by (×) are where increased water permeability was observed when expressed in yeast, but no change to CO_2 permeability was observed. For *N tabacum*, AQP1 is assigned to PIP1;2. Multiple symbols show instances where there are conflicting reports. The assignment of the same PIP number between species does not necessarily imply that the genes are orthologous.

Plant	References		PIP1							PIP2							
			1;1	1;2	1;3	1;4	1;5	1;6		2;1	2;2	2;3	2;4	2;5	2;6	2;7	2;8
Arabidopsis thaliana	1	H_2O	✓	w, ×	✓					✓	✓	✓				✓	
	2	CO_2		Y						✓		×					
Beta vulgaris	3	H_2O	+							✓	✓						
Fragaria ananassa	4	H_2O	+							✓							
Helianthemum almeriense	5	H_2O	×							Y							
		CO_2	Y							×							
Hevea brasiliensis	6	H_2O								✓		✓					
Hordeum vulgare	7	H_2O		+, Y	w	+				✓	✓	✓	✓	✓		✓	✓
	8	CO_2								✓	✓	✓	×	✓			
Juglans regia	9	H_2O								✓							
Lilium longiflorum	10	H_2O	✓														
Mimosa pudica	11	H_2O	+							✓							

Nicotiana tabacum	12	H_2O	+	w,×, ✓						✓							
	13	CO_2		Y,P, ✓						P							
Oryza sativa	14	H_2O	w		w					✓	✓	✓	✓	✓		✓	
	15	CO_2								×							
Phaseolus vulgaris	16	H_2O										✓					
Rosa hybrida	17	H_2O	+							✓							
Raphanus sativa	18	H_2O		w	w					✓	✓	✓					
Spinacia oleracea	19	H_2O								✓							
Samanea saman	20	H_2O								SsAQP2							
Tulipa gesneriana	21	H_2O									✓						
Vitis vinifera	22	H_2O	+			×		+		✓	✓	✓,×	✓				
Zea mays	23	H_2O	+	+			+	Y		✓				✓			
	24	CO_2					Y	Y									

References

1. (Daniels *et al.*, 1994, Heckwolf *et al.*, 2011, Kammerloher *et al.*, 1994, Soto *et al.*, 2008, Weig *et al.*, 1997)
2. (Heckwolf *et al.*, 2011, Uehlein *et al.*, 2012b, Wang *et al.*, 2016)
3. (Bellati *et al.*, 2010, Jozefkowicz *et al.*, 2013)

4. (Alleva *et al.*, 2010)
5. (Navarro-Rodenas *et al.*, 2013)
6. (An *et al.*, 2015)
7. (Besse *et al.*, 2011, Horie *et al.*, 2011, Katsuhara *et al.*, 2002, Shibasaka *et al.*, 2012)
8. (Mori *et al.*, 2014)
9. (Sakr *et al.*, 2003)
10. (Ding *et al.*, 2004)
11. (Temmei *et al.*, 2005)
12. (Biela *et al.*, 1999, Mahdieh *et al.*, 2008, Otto *et al.*, 2010, Sade *et al.*, 2010, Siefritz *et al.*, 2002)
13. (Ding *et al.*, 2013, Otto *et al.*, 2010, Uehlein *et al.*, 2003, Uehlein *et al.*, 2012a)
14. (Ding *et al.*, 2016, Ding *et al.*, 2013, Li *et al.*, 2008, Li *et al.*, 2000, Lian *et al.*, 2004, Sakurai *et al.*, 2008, Sakurai *et al.*, 2005, Zhang *et al.*, 2010)
15. (Ding *et al.*, 2013)
16. (Zhou *et al.*, 2007)
17. (Chen *et al.*, 2013, Ma *et al.*, 2008, Postaire *et al.*, 2010)
18. (Suga & Maeshima, 2004)
19. (Johansson *et al.*, 1998)
20. (Moshelion *et al.*, 2002)
21. (Azad *et al.*, 2008)
22. (Sabir *et al.*, 2014, Shelden *et al.*, 2009, Vandeleur *et al.*, 2009)
23. (Bienert *et al.*, 2012, Chaumont *et al.*, 2000, Fetter *et al.*, 2004, Heinen *et al.*, 2014)
24. (Heinen *et al.*, 2014)

Table 2. Increased permeability of *Xenopus* oocytes to water when particular PIP1s (+) are co-expressed together with PIP2s (✓). The sole exception so far is ZmPIP1;1, where co-expression with ZmPIP1;2 (c) rendered the PIP1 active. Where several different PIP1 genes have been investigated for a given species, a separate line is used to identify interactions for each PIP1.

Plant	Reference	PIP1							PIP2							
		1;1	1;2	1;3	1;4	1;5	1;6		2;1	2;2	2;3	2;4	2;5	2;6	2;7	2;8
Beta vulgaris	(Bellati *et al.*, 2010, Jozefkowicz *et al.*, 2013)	+								✓						
Fragaria ananassa	(Alleva *et al.*, 2010)	+							✓							
Hordeum vulgare	(Horie *et al.*, 2011)		+						✓	✓	✓	✓	✓			
					+				✓	✓	✓	✓	✓			
Mimosa pudica	(Temmei *et al.*, 2005)	+							✓							
Nicotiana tabacum	(Mahdieh *et al.*, 2008)	+							✓							
Rosa hybrida	(Chen *et al.*, 2013)	+							✓							
Vitis vinifera	(Vandeleur *et al.*, 2009)	+								✓						
Zea mays	(Fetter *et al.*, 2004)	c	c													
			+						✓		At	✓	✓			
	(Bienert *et al.*, 2012)		+										✓			
	(Heinen *et al.*, 2014)					+			✓							
							+		✓							

Table 3. Summary of aquaporins that have been used in attempts to engineer plants with improved responses to different abiotic conditions including drought, salt or cold stress and tolerance when exposed to heavy metals or pathogen infection. Symbols represent improved growth (✓), no difference to WT (=), or negative effects on growth (X).

			Condition					
Gene	**Promoter**	**Host**	**Normal**	**Drought**	**Salt**	**Cold**	**Other**	**Reference**
AcNIP5;1	35S	Arabidopsis		✓	X			(Yu *et al.*, 2015)
AcPIP2	35S	Arabidopsis	✓	X	✓			(Li *et al.*, 2015a)
AtPIP1;2	35S	Tobacco	✓	X	=			(Aharon *et al.*, 2003)
AtPIP1;4	35S	Tobacco		X		✓		(Jang *et al.*, 2007)
AtPIP1;4	35S	Arabidopsis	✓	X		=	✓ Bacterial infection	(Jang *et al.*, 2007, Li *et al.*, 2015b, Tian *et al.*, 2016)
AtPIP2;5	35S	Tobacco		X		✓		(Jang *et al.*, 2007)
AtPIP2;5	35S	Arabidopsis		X		=		(Jang *et al.*, 2007)
AtTIP5;1	35S	Arabidopsis					✓ Boron	(Pang *et al.*, 2010)
BjPIP1	35S	Tobacco		✓			✓ Cadmium	(Zhang *et al.*, 2008)
BnPIP1	35S	Tobacco		✓				(Yu *et al.*, 2005)
FaPIP2;1		Arabidopsis		✓				(Zhuang *et al.*, 2015)
GmPIP1;6	35S	Tobacco	=		✓			(Zhou *et al.*, 2014)
HvPIP2;1	35S	Rice	✓					(Hanba *et al.*, 2004)
HvPIP2;1	35S	Rice			X			(Katsuhara *et al.*, 2003)
JcPIP2;7	35S	Arabidopsis	=	✓	✓			(Khan *et al.*, 2015)
JcTIP1;3	35S	Arabidopsis	=	✓	✓			(Khan *et al.*, 2015)
MaPIP1;1	35S	Arabidopsis		✓	✓			(Xu *et al.*, 2014)
McMIPB	35S	Tobacco	✓					(Kawase *et al.*, 2013)

MusaPIP1;2	Ubiquitin	Banana		✓	✓	✓		(Sreedharan *et al.*, 2013)
MusaPIP2;6	Ubiquitin	Tobacco	=		✓			(Sreedharan *et al.*, 2015)
NtAQP1	35S	Arabidopsis	✓		✓			(Sade *et al.*, 2014a, Sade *et al.*, 2010)
NtAQP1	35S	Tomato	✓					(Kelly *et al.*, 2014)
NtAQP1	35S	Tomato	✓		✓			(Sade *et al.*, 2010)
NtAQP1	35S	Tobacco	✓					(Uehlein *et al.*, 2003)
OsPIP1;1/2;2	35S	Arabidopsis	=		✓			(Guo *et al.*, 2006)
OsPIP2;4/2;6/2;7	actin2	Arabidopsis					✓ Arsenic	(Mosa *et al.*, 2012)
PgTIP1	35S	Arabidopsis	✓	✓	✓	X		(Peng *et al.*, 2007)
PtdPIP2;5	Ubiquitin	Poplar	=			✓		(Ranganathan *et al.*, 2016)
RsPIP2;1	35S	Eucalyptus	✓		=			(Tsuchihira *et al.*, 2010)
SlTIP2;2	EVO205	Tomato		✓	✓			(Sade *et al.*, 2009)
SpAQP1	35S	Tobacco	=		✓			(Chang *et al.*, 2016)
TaAQP7	35S	Tobacco		✓				(Zhou *et al.*, 2012)
TaAQP7	35S	Tobacco				✓		(Huang *et al.*, 2014)
TaAQP8	35S	Tobacco			✓			(Hu *et al.*, 2012)
TaNIP	35S	Arabidopsis			✓			(Gao *et al.*, 2010)
TdPIP1;1/2;1	35S	Tobacco	✓		✓			(Ayadi *et al.*, 2011)
TsTIP1;2	35S	Arabidopsis		✓	✓			(Wang *et al.*, 2014)
VfPIP1	35S	Arabidopsis		✓				(Cui *et al.*, 2008)

Figure Legends

Figure 1. Aquaporin diversity across the green plant (Viridiplantae) kingdom. The phylogenetic tree is based on reconstructions from (Leliaert *et al.*, 2012, Ruhfel *et al.*, 2014), with major divisions listed at their respective evolutionary points. Polyploidy events (red stars) were obtained from summaries provided in (Mühlhausen & Kollmar, 2013) and CoGepedia https://genomevolution.org/wiki/index.php/Plant_paleopolyploidy. Suspected genome doubling in Rubber tree (Zou *et al.*, 2015a, Zou *et al.*, 2016); consistent with the higher frequency of aquaporin genes compared to close relatives of Physic nut and Castor bean. Modifications from initial classification: *Physcomitrella patens*; removed unlikely third PIP group (PIP3) as PpPIP3;1 is likely non-functional (Danielson & Johanson, 2008). *Citrus sinensis*; removed CsTIP1;3, CsTIP5;1 and CsTIP6;1 as these were all substantially truncated proteins. *Equisetum arvense*; reassigned EaTIP1;1 and EaTIP2;1 to EaTIP3;1 and EaTIP3;2 in accordance with (Abascal *et al.*, 2014). *Picea glauca*; reassigned PgTIP1s as PgTIP3s in accordance with (Abascal *et al.*, 2014). *Populus trichocarpa*; reassigned PtXIPs in accordance with (Lopez *et al.*, 2012) and PtNIPs in accordance with (Zou *et al.*, 2015b). *Solanum lycopersicum*; removed two SlNIPs due to substantial deletions. Noted limitations in data: *Equisetum arvense* information derived from root transcriptome only (Gregoire *et al.*, 2012). *Triticum aestivum*; unlikely to be an exhaustive search (Forrest & Bhave, 2008, Pandey *et al.*, 2013). Currently there is a lack of aquaporin family data in the charophyta. Aquaporin data in this primitive evolutionary important plant lineage could contribute significantly to our understanding of early plant aquaporin evolution.

Figure 2. Diagrammatic illustration of water flow from the soil through the plant to the atmosphere and CO_2 diffusion between the leaf and atmosphere. Water can flow either along cell walls via the apoplast or through cells via plasmodesmata in the symplast. Water needs to cross plasma membranes at several points along the way through aquaporins, which allow bidirectional flow. The combination of apoplastic and symplastic flow enabled by entry and exit via aquaporins is termed transcellular flow. Water entry and exit from the vascular system is isolated from apoplastic flow by the endodermis in roots and the bundle sheath in leaves. Mature guard cells have no symplastic connection to adjacent epidermal cells. Not shown is the cuticle that lines the external surface of the epidermis making it impermeable to water and CO_2. Leaf mesophyll and stomatal guard cells have aquaporins that enhance the permeability of the plasma membrane and chloroplast envelope to CO_2.

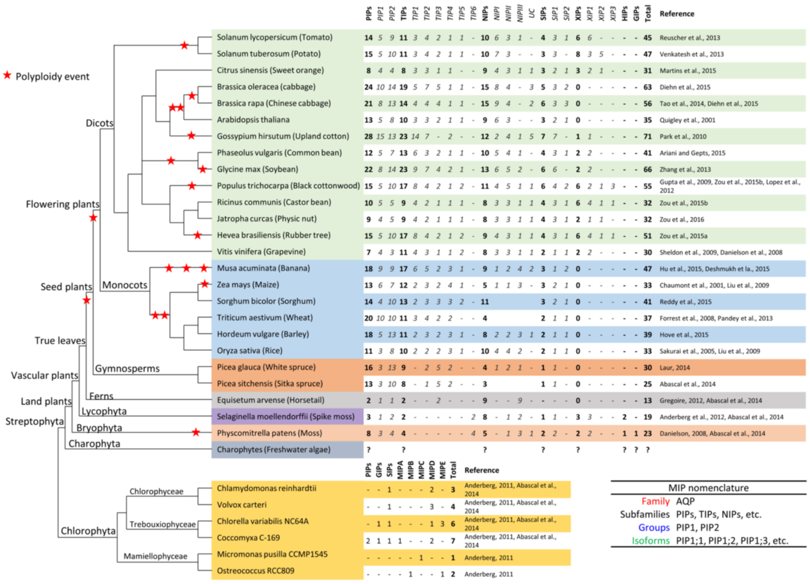
Figure 3. Impact of varying mesophyll conductance on CO_2 assimilation rate and transpiration efficiency. Two scenarios are modelled: constant stomatal conductance (0.3 mol H_2O m^{-2} s^{-1}, solid curves), or constant C_i (280 µbar, dashed red curves). A Rate of CO_2 assimilation, B drawdown in CO_2 partial pressure between the intercellular airspaces (C_i) and the sites of carboxylation in the chloroplast (C_c), C transpiration rate, D transpiration efficiency (rate of CO_2 assimilation/transpiration rate). Model curves assume Rubisco activity of 100 µmol m^{-2} s^{-1}, respiration rate 1 µmol m^{-2} s^{-1}, Γ^* 35.5 µbar, K_{21} 550 µbar, leaf to air vapour pressure difference 12 mbar (von Caemmerer *et al.*, 1994, von Caemmerer & Farquhar, 1981). Also shown in panel B are three examples where mesophyll conductance has been altered by antisense / knockout (AS) or overexpression (OE), respectively, of an aquaporin compared to their respective wild type (WT): rice (Hanba

et al., 2004), tobacco (Flexas *et al.*, 2006), *Arabidopsis* (Li *et al.*, 2015b). Mesophyll conductance (0.05 – 0.6 mol m^{-2} s^{-1} bar^{-1}) typically scales with photosynthetic capacity which results in similar CO_2 drawdowns across the mesophyll (C_i - C_c, typically between 50 -150 µbar under high irradiance).

Figure 4. Diagram of a PIP aquaporin showing key amino acid residues involved in gating, substrate specificity and dimerization. Amino acids are numbered in reference to an alignment with SoPIP2;1; the numbering of the equivalent residues in the actual tested PIPs can be found in Supplementary Tables 1 and 2. Supplementary Tables 1 and 2 also provide summaries of the functional role of these residues. Further details of the interactions between residues involved in gating can be found in Supplementary Figure 1. Dimerisation of two monomers occurs through a disulphide bridge at C69 (Bienert *et al.*, 2012) and other interactions associated with Loop A and the transmembrane helices (Jozefkowicz *et al.*, 2013, Yoo *et al.*, 2016). The mutation I244M at the end of helix 6 stops permeability to CO_2 without affecting permeability to water for HvPIP2;3 and HvPIP2;4 (Mori *et al.*, 2014).

Summary statement

Aquaporins are intrinsic membrane proteins encoded by a multigene family that function to increase membrane permeability to water, CO2 and other molecules. There are many levels of regulation (e.g. diurnal expression, membrane targeting, tetramer composition, gating) that result in highly dynamic and tissue specific control of permeability. Owing to the ease of measurement, aquaporins associated with water permeability have been more extensively characterised than those facilitating CO2 transport. Manipulation of aquaporins is actively being explored in efforts to improve plant performance with respect to plant water relations, stomatal function and photosynthesis.



	PIPs	PIP1	PIP2	TIPs	TIP1	TIP2	TIP3	TIP4	TIP5	TIP6	NIPs	NIPI	NIPII	NIPIII	UC	SIPs	SIP1	SIP2	XIPs	XIP1	XIP2	XIP3	HIPs	GIPs	Total	Reference
Solanum lycopersicum (Tomato)	14	5	9	11	3	4	2	1	1	-	10	6	3	1	-	4	3	1	6	6	-	-	-	-	45	Reuscher et al., 2013
Solanum tuberosum (Potato)	15	5	10	11	3	4	2	1	1	-	10	7	3	-	-	3	3	-	8	3	5	-	-	-	47	Venkatesh et al., 2013
Citrus sinensis (Sweet orange)	8	4	4	8	3	3	1	1	-	-	9	4	3	1	1	3	2	1	3	2	1	-	-	-	31	Martins et al., 2015
Brassica oleracea (cabbage)	24	10	14	19	5	7	5	1	1	-	15	8	4	-	3	5	3	2	0	-	-	-	-	-	63	Diehn et al., 2015
Brassica rapa (Chinese cabbage)	21	8	13	14	4	4	4	1	1	-	15	9	4	-	2	6	3	3	0	-	-	-	-	-	56	Tao et al., 2014, Diehn et al., 2015
Arabidopsis thaliana	13	5	8	10	3	3	2	1	1	-	9	6	3	-	-	3	2	1	0	-	-	-	-	-	35	Quigley et al., 2001
Gossypium hirsutum (Upland cotton)	28	15	13	23	14	7	-	2	-	-	12	2	4	1	5	7	7	-	1	1	-	-	-	-	71	Park et al., 2010
Phaseolus vulgaris (Common bean)	12	5	7	13	6	3	2	1	1	-	10	5	4	1	-	4	3	1	2	2	-	-	-	-	41	Ariani and Gepts, 2015
Glycine max (Soybean)	22	8	14	23	9	7	4	2	1	-	13	6	5	2	-	6	6	-	2	2	-	-	-	-	66	Zhang et al., 2013
Populus trichocarpa (Black cottonwood)	15	5	10	17	8	4	2	1	2	-	11	4	5	1	1	6	4	2	6	2	1	3	-	-	55	Gupta et al., 2009, Zou et al., 2015b, Lopez et al., 2012
Ricinus communis (Castor bean)	10	5	5	9	4	2	1	1	1	-	8	3	3	1	1	4	3	1	6	4	1	1	-	-	32	Zou et al., 2015b
Jatropha curcas (Physic nut)	9	4	5	9	4	2	1	1	1	-	8	3	3	1	1	4	3	1	2	1	1	-	-	-	32	Zou et al., 2016
Hevea brasiliensis (Rubber tree)	15	5	10	17	8	4	2	1	2	-	9	4	3	1	1	4	3	1	6	4	1	1	-	-	51	Zou et al., 2015a
Vitis vinifera (Grapevine)	7	4	3	11	4	3	1	1	2	-	8	3	3	1	1	2	1	1	2	2	-	-	-	-	30	Sheldon et al., 2009, Danielson et al., 2008
Musa acuminata (Banana)	18	9	9	17	6	5	2	3	1	-	9	1	2	4	2	3	1	2	0	-	-	-	-	-	47	Hu et al., 2015, Deshmukh et la., 2015
Zea mays (Maize)	13	6	7	12	2	3	2	4	1	-	5	1	1	3	-	3	2	1	0	-	-	-	-	-	33	Chaumont et al., 2001, Liu et al., 2009
Sorghum bicolor (Sorghum)	14	4	10	13	2	3	3	3	2	-	11					3	2	1	0	-	-	-	-	-	41	Reddy et al., 2015
Triticum aestivum (Wheat)	20	10	10	11	3	4	2	2	-	-	4					2	1	1	0	-	-	-	-	-	37	Forrest et al., 2008, Pandey et al., 2013
Hordeum vulgare (Barley)	18	5	13	11	2	3	2	3	1	-	8	2	2	3	1	2	1	1	0	-	-	-	-	-	39	Hove et al., 2015
Oryza sativa (Rice)	11	3	8	10	2	2	2	3	1	-	10	4	4	2	-	2	1	1	0	-	-	-	-	-	33	Sakurai et al., 2005, Liu et al., 2009
Picea glauca (White spruce)	16	3	13	9	-	2	5	2	-	-	4	1	2	1	-	1	1	-	0	-	-	-	-	-	30	Laur, 2014
Picea sitchensis (Sitka spruce)	13	3	10	8	-	1	5	2	-	-	3					1	1	-	0	-	-	-	-	-	25	Abascal et al., 2014
Equisetum arvense (Horsetail)	2	1	1	2	-	-	2	-	-	-	9	-	-	9	-	-	-	-	0	-	-	-	-	-	13	Gregoire, 2012, Abascal et al., 2014
Selaginella moellendorffii (Spike moss)	3	1	2	2	-	-	-	-	-	2	8	-	1	2	-	1	1	-	3	3	-	-	2	-	19	Anderberg et al., 2012, Abascal et al., 2014
Physcomitrella patens (Moss)	8	3	4	4	-	-	-	-	-	4	5	-	1	3	1	2	2	-	2	2	-	-	1	1	23	Danielson, 2008, Abascal et al., 2014
Charophytes (Freshwater algae)	?			?							?					?			?				?	?	?	

	PIPs	GIPs	SIPs	MIPA	MIPB	MIPC	MIPD	MIPE	Total	Reference
Chlamydomonas reinhardtii	-	-	1	-	-	-	2	-	3	Anderberg, 2011, Abascal et al., 2014
Volvox carteri	-	-	1	-	-	-	3	-	4	Anderberg, 2011, Abascal et al., 2014
Chlorella variabilis NC64A	-	1	1	-	-	-	1	3	6	Anderberg, 2011, Abascal et al., 2014
Coccomyxa C-169	2	1	1	1	-	-	2	-	7	Anderberg, 2011, Abascal et al., 2014
Micromonas pusilla CCMP1545	-	-	-	-	-	1	-	-	1	Anderberg, 2011
Ostreococcus RCC809	-	-	-	-	1	-	-	1	2	Anderberg, 2011

MIP nomenclature	
Family	AQP
Subfamilies	PIPs, TIPs, NIPs, etc.
Groups	PIP1, PIP2
Isoforms	PIP1;1, PIP1;2, PIP1;3, etc.

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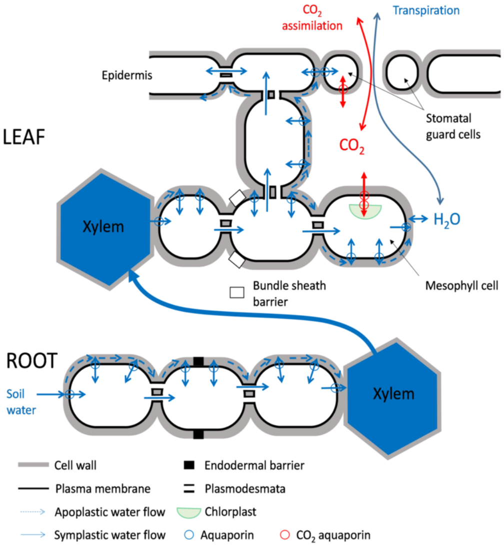

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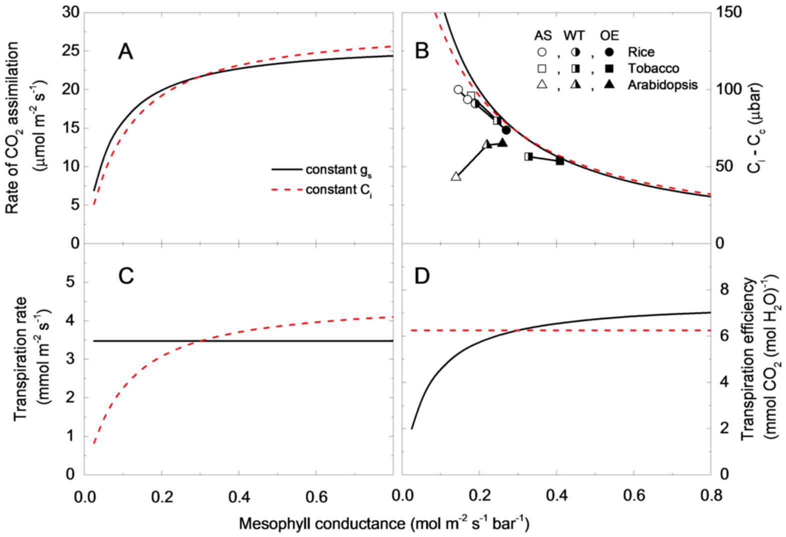

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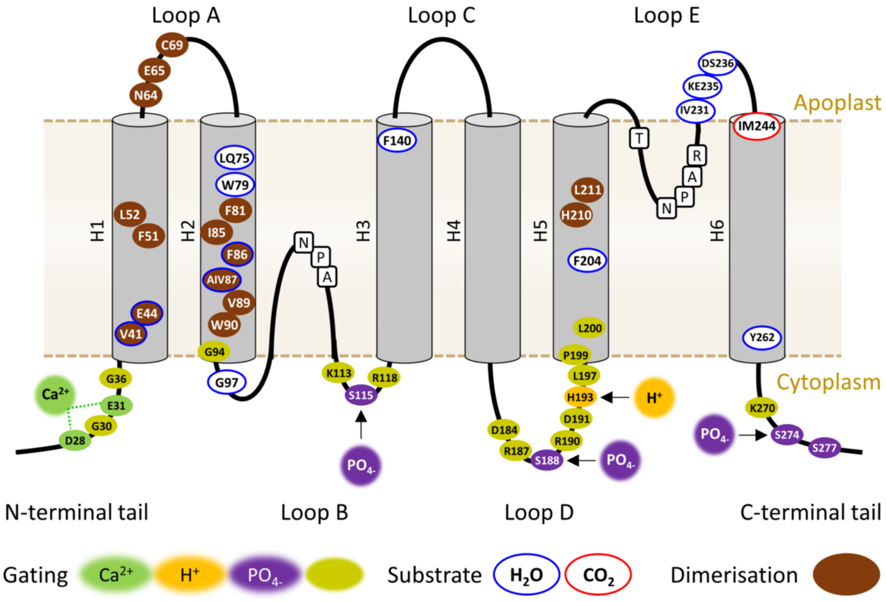

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